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(54) Title: SUPPRESSION OF ENDOGENOUS IGFBP-2 TO INHIBIT CANCER**(57) Abstract**

The present invention concerns the role of IGFBP-2 in cancer. Cancers that are dependent on IGFBP-2 would benefit from therapies targeting IGFBP-2. The invention discloses methods for inhibiting IGFBP-2 dependent cancers by modulating IGFBP-2 activity. Modulation can be achieved by decreasing the amounts of IGFBP-2 protein or IGFBP-2 activity, as well as other polypeptides that interact with or modify IGFBP-2. Preferred modulators of IGFBP-2 include antibodies specific for IGFBP-2 and antisense or ribozymes specific for IGFBP-2 nucleic acid. These modulators are useful for treating cancers derived from any tissue, with cancer of the prostate being preferred. Methods of treating a subject with cancer are disclosed as well as compositions of IGFBP-2 modulators and kits containing such modulators.

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DESCRIPTION**SUPPRESSION OF ENDOGENOUS IGFBP-2 TO INHIBIT CANCER****BACKGROUND OF THE INVENTION**

5 This application claims priority to U.S. Serial No. 60/134,581, which is a provisional application filed on May 17, 1999 that is hereby incorporated by reference in its entirety. The U.S. government may own rights in this invention pursuant to grant numbers CA72992 and CA60087 from the National Institutes of Health.

10 **1. FIELD OF THE INVENTION**

The present invention relates generally to the fields of biochemistry and cancer. More particularly, it concerns Insulin-like Growth Factor Binding Protein 2 (IGFBP-2) and its relevance to cancer.

15 **2. DESCRIPTION OF RELATED ART**

Prostate cancer is the most common non-skin cancer diagnosed in men in the United States. With about 184,000 new cases found, and about 39,200 deaths attributed to prostate cancer each year, it is second only to lung cancer as the most common cause of death from malignancy. The cause of prostate cancer is unknown, 20 and the tumor often remains clinically silent until it has reached an advanced stage in men over 60 years of age. The methods currently used to diagnose prostate cancer, such as physical exam, monitoring Prostate-Specific Antigen (PSA) levels, tissue biopsy and ultrasound and bone scans, are restricted in both sensitivity and specificity. Since the survival of prostate cancer patients depends on early detection and treatment, 25 novel strategies for early diagnosis and treatment of the disease must be developed.

The Insulin-like Growth Factor (IGF) system is a multi-component network of molecules (Juul *et al.*, 1995) involved in the regulation of cell growth in a variety of organs (Rajaram *et al.* 1997), including the prostate (Cohen *et al.*, 1994; Thrasher *et al.*, 1996). The IGF binding proteins (IGFBPs) are a family of high affinity binding

proteins, which regulate IGF availability to the receptors, and are now being recognized as a new class of growth modulators (Cohen and Rosenfeld, 1994). IGFBP-2 is elevated in the serum of patients with prostate cancer (Ho and Baxter, 1997; Kanety *et al.*, 1993; Figueroa *et al.*, 1998), but not in benign prostatic hyperplasia (BPH) (Ho and Baxter, 1997; Cohen *et al.*, 1993). Elevation of IGFBP-2 is related to the stage of the tumor and serum prostate-specific antigen (PSA) levels, suggesting a positive correlation between IGFBP-2 and prostate cancer. Increment in serum IGFBP-2 correlates positively with severity of many other cancers as well (Reeve *et al.*, 1992a and 1992b; Karasik *et al.*, 1994; Kanety *et al.*, 1996; Wang *et al.*, 1997; Flyvbjerg *et al.*, 1997; Menouny *et al.*, 1998), but the role of IGFBP-2 in carcinogenesis was unexplored. It is not clear whether the increase in serum IGFBP-2 seen in prostate cancer is causative of progression of the cancer or simply a passive marker.

15 Insights into improved therapies for cancers are needed. While there are available therapies for various cancers, including chemotherapy, radiotherapy, and immunotherapy, novel approaches showing improved success must be developed. Often times, it is a unique combination of therapies that results in the best efficacy towards a given cancer. In addition to the roles of growth factors and their receptors in

20 cancer, the roles of growth factor serum binding proteins in cancer needs to be addressed.

SUMMARY OF THE INVENTION

25 The present invention discloses a method of inhibiting an IGFBP-2 dependent cancer cell comprising reducing IGFBP-2 activity level with an IGFBP-2 modulator. Inhibiting an IGFBP-2 dependent cancer cell includes altering proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion.

30

The IGFBP-2 modulator may decrease the amount of IGFBP-2, inhibit expression, transcription, translation, secretion, or inhibit the functional activity of IGFBP-2. The IGFBP-2 modulator may be an agonist or antagonist of IGFBP-2.

5 The modulator of IGFBP-2 is a nucleic acid containing a promoter operably linked to a IGFBP-2 gene segment. In preferred embodiments, the IGFBP-2 gene segment is positioned, in reverse orientation, under the control of a promoter that directs expression of an antisense product. In other embodiments, the nucleic acid encodes a ribozyme specific for an RNA transcript of IGFBP-2 in a cell expressing an
10 RNA transcript of IGFBP-2. The promoter that is operably linked to a gene segment that encodes IGFBP-2 functions in a mammalian cell.

15 The nucleic acid segment containing a promoter operably linked to a IGFBP-2 gene segment may be located on a vector, such as a plasmid vector. In other embodiments, the vector is a viral vector selected from the group consisting of adenovirus, retrovirus, herpes virus, adeno-associated virus, and vaccinia virus. The nucleic acid may be packaged in a virus particle. The promoter that is operably linked to a gene segment that encodes IGFBP-2 functions in a mammalian cell.

20 In preferred embodiments, the IGFBP-2 modulator is an antibody composition comprising an antibody that recognizes IGFBP-2. The antibody composition may comprise a monoclonal antibody, polyclonal antibodies, or a humanized antibody.

25 The IGFBP-2 modulator may be at least one polypeptide that interacts with IGFBP-2, and may comprises IGF-I, IGF-II, IFGBP-2 receptor, or a fusion protein or fragment thereof. The IGFBP-2 modulator may also be a polypeptide involved in post-translational modification of IGFBP-2.

30 The IGFBP-2 dependent cell may be in cell culture or in an animal. The animal may be a mammal and may be a human. The IGFBP-2 dependent cell may be derived from the brain (*e.g.*, glioblastoma, astrocytoma, oligodendroglioma, ependymomas), neurofibrosarcoma, meningia, lung, liver, spleen, kidney, lymph node, pancreas, small

intestine, blood cells, colon or colorectal region, stomach, thyroid, breast, endometrium, prostate, testes, ovary, skin, head and neck, esophagus, bone marrow, blood or other tissue. In preferred embodiments, the cell is derived from the prostate.

5 Also disclosed are methods of treating a subject with cancer comprising administering to the subject an effective amount of a IGFBP-2 modulator. In preferred embodiments, the subject is a human. The IGFBP-2 modulator may be an antibody composition comprising an antibody that recognizes IGFBP-2. The antibody composition may comprises a monoclonal antibody, polyclonal antibodies, or a
10 10 humanized antibody. Alternatively, the modulator may be a ribozyme or antisense sequence complementary to the IGFBP-2 gene.

15 Treating a subject with cancer may result in killing a cancer cell, inhibiting the growth of a cancer cell, inducing apoptosis of a cancer cell, inhibiting metastatic potential of a cancer cell, reducing tumor burden, or inducing tumor regression. The cancer may be a cancer of the brain (*e.g.*, glioblastoma, astrocytoma, oligodendrogloma, ependymomas), neurofibrosarcoma, meningia, lung, liver, spleen, kidney, lymph node, pancreas, small intestine, blood cells, colon or colorectal region, stomach, thyroid, breast, endometrium, prostate, testes, ovary, skin, head and neck,
20 20 esophagus, bone marrow, blood or other tissue. In preferred embodiments, the cancer is prostate cancer.

25 The method of treating a subject with cancer may also comprise treating the subject with a second agent, wherein the second agent is a second modulator of IGFBP-2, a therapeutic polypeptide, nucleic acid encoding a therapeutic polypeptide, a chemotherapeutic agent, or a radiotherapeutic agent.

30 Also disclosed is a method of screening a candidate substance for anti-tumor activity comprising the steps of contacting a first IGFBP-2 dependent cell with a candidate substance, and comparing one or more characteristics of the first cell in the presence of the candidate substance with one or more characteristics of a second IGFBP-2 dependent cell in the absence of the candidate substance. Characteristic that

can be examined may be selected from the group consisting of IGFBP-2 expression, proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, and tumor invasion. The IGFBP-2 dependent cell may be contacted *in vitro* or *in vivo*.

5

Other embodiments of the present invention include therapeutic and prophylactic methods to treat or prevent cancer. The cancer may involve a tumor. In some aspects, the methods of treating a cancer patient involve administering to the patient a composition that contains a peptide having at least 8 contiguous amino acids 10 from the amino acid sequence of human IGFBP-2 (SEQ ID NO:2) in an amount effective to convey a therapeutic benefit on the patient. The term "therapeutic benefit" used throughout this application refers to anything that promotes or enhances the well-being of the subject with respect to the medical treatment of his condition, which includes treatment of cancer or other hyperproliferative diseases. A list of 15 nonexhaustive examples of such benefit includes extension of the subject's life by any period of time, decrease or delay in the neoplastic development of the disease, decrease in hyperproliferation, reduction in tumor growth or size, delay of metastases, reduction in cancer cell or tumor cell proliferation rate, and a decrease in pain to the subject that can be attributed to the subject's condition. In the context of the invention, the 20 therapeutic benefit conveyed on the patient may generally involve inhibition of an IGFBP-2-dependent cancer cell, whose characteristics have been discussed above.

In some aspects of the present invention, the preventative and therapeutic methods involve a composition that includes an adjuvant in addition to an IGFBP-2 peptide or polypeptide. The adjuvant may or may not be linked to the peptide or 25 polypeptide. If the adjuvant is linked to the IGFBP-2 peptide or polypeptide, they may be covalently linked. It is contemplated that the IGFBP-2 peptide may be of any length, though the peptide of SEQ ID NO:3 has been used to generate antibodies. A peptide that contains at least 8 contiguous amino acids from SEQ ID NO:3 may be 30 employed in the methods of the present invention, as well as a peptide comprising SEQ ID NO:3.

It is also contemplated that the therapeutic methods of the invention may be employed in combination with another anti-cancer treatment such as chemotherapy, radiotherapy, surgery, hormone therapy, or gene therapy, which may be administered before administration of the IGFBP-2 directed agent, which includes an IGFPB-2 peptide that confers a therapeutic benefit, as well as after or at the same time as the IGFBP-2 directed agent.

The preventative methods of the invention rely upon the immune system to effect a response against IGFBP-2. An immune response includes a humoral as well as 10 a cellular or cell-mediated response. Thus, it is contemplated that a subject could be immunized with an IGFBP-2 peptide or polypeptide to generate a level of humoral and/or cellular immunity against IGFBP-2. Multiple immunizations or boosters may be given to a subject to maintain the immune response.

15 Also disclosed are pharmaceutical compositions comprising a recombinant vector containing a promoter operably linked to a IGFBP-2 gene segment. The pharmaceutical composition may have the IGFBP-2 gene segment positioned, in reverse orientation, under the control of a promoter that directs expression of an antisense product. Alternatively, the pharmaceutical composition may be a 20 recombinant vector expressing a ribozyme specific for an RNA transcript of IGFBP-2 in a cell expressing an RNA transcript of IGFBP-2.

Finally, kits are disclosed comprising a suitable container and at least a first antibody composition that recognizes IGFBP-2 or a kit comprising a suitable container 25 and at least a recombinant vector containing a promoter operably linked to a nucleic acid segment that encodes IGFBP-2.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also 30 consistent with the meaning of "one or more," "at least one," and "one or more than one."

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various 5 changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF DRAWINGS

10 The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

15 **FIG. 1A, FIG. 1B, FIG. 1C, and FIG. 1D.** Effect of IGFBP-2 and IGFBP-2 antibody (Ab) on the growth of LNCap and DU145 prostate cancer cells. LNCap and DU145 cells were treated with different doses of IGFBP-2 or IGFBP-2 Ab, with or without IGF-I (250 ng/ml), and growth was measured by counting the total number of cells (FIG. 1A. - LNCap; FIG. 1B. - DU145), or by an MTT assay (FIG. 1C.- LNCap; 20 FIG. 1D.- DU145). The Y axis of panels FIG. 1C. and FIG. 1D. represent Optical Density at 560 nm. In the sections designated by "Serum" and "Control," the three bars represent growth of Control cells in 1%, 5% and 10% concentrations of fetal calf serum (FCS).

25 **FIG. 2.** Tumorigenic potential of DU145 cells grown in the presence of IGFBP-2 or IGFBP-2 Ab, determined in a soft agar clonogenic assay. The total number of colonies/well in wells seeded with IGFBP-2 Ab was less than 1-5% of the numbers in wells seeded with IGFBP-2.

30 **FIG. 3.** Growth of AS-clones of LNCap cells in the presence of different concentrations of serum. Growth was measured in terms of optical density

(O.D.) by an MTT assay. Results are mean \pm SEM of 8 observations with the representative clones.

5 **FIG. 4.** IGF-I protein levels in CM collected from Control, IGF and
IGFBP-2 treated cultures of DU145 cells. The levels of IGF increase 3-4 fold times
following treatment with IGF. However, the levels of IGF are undetectable following
treatment with IGFBP-2 antibody.

10 **FIG. 5.** Initiation of DU145-generated tumors in nude mice following
inoculation with cells mixed with non-immune IgG (Control) and cells mixed with anti-
IGFBP-2 IgG (Treated). The initiation of tumors was delayed significantly ($p<0.001$)
when cells are co-inoculated with anti-IGFBP-2.

15 **FIG. 6.** Weight comparison of tumors generated in male athymic nude
mice from wild type (WT) and anti-IGFBP-2 IgG mixed cells (Cells + IGFBP-2
antibody ("BP-2 Ab")). Tumors generated from WT cells are significantly ($p<0.001$)
larger than those generated from cells mixed with anti-IGFBP-2 IgG.

20 **FIG. 7.** Effect of anti-IGFBP-2 IgG on the growth of DU145 prostatic
tumors as measured by tumor weight. Group I: Inoculated with untreated cells (2×10^6 cells)
on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 30 μ g anti-IGFBP-2 IgG on left.
Group II: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 60 μ g
anti-IGFBP-2 IgG on left. Group III: Inoculated with untreated cells (2×10^6 cells)
25 on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 120 μ g
anti-IGFBP-2 IgG on left.

30 **FIG. 8.** Effect of anti-IGFBP-2 IgG on total tumor weight in control
and treated groups of animals. Animals of Group A were inoculated on the left side
with 2×10^6 DU145 cells mixed with 12 μ g of non-immune IgG, and with an equal

concentration of untreated cells on the right side. Group B animals were inoculated on both sides with untreated DU145 cells.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

5

The present invention concerns the role of IGFBP-2 in cancer. Cancers that are dependent on IGFBP-2 would benefit from therapies targeting IGFBP-2. The invention discloses methods for inhibiting IGFBP-2 dependent cancers by modulating IGFBP-2 activity. Modulation can be achieved by decreasing the amounts of IGFBP-2 10 protein or IGFBP-2 activity, as well as other polypeptides that interact with or modify IGFBP-2. Preferred modulators of IGFBP-2 include antibodies specific for IGFBP-2 and antisense or ribozymes specific for IGFBP-2 nucleic acid. These modulators are useful for treating cancers derived from any tissue, with cancer of the prostate being preferred. Methods of treating a subject with cancer are disclosed as well as 15 compositions of IGFBP-2 modulators and kits containing such modulators.

The present invention is directed at methods for reducing IGFBP-2 activity in such a way as to affect an IGFBP-2 dependent cell. The effect on the IGFBP-2 dependent cell can be an alteration in proliferation, metastasis, contact inhibition, soft 20 agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion. IGFBP-2 activity can be reduced *in vitro* or *in vivo* by a number of methods as detailed below.

Three general approaches are described for decreasing the amount of IGFBP-2 25 activity. These are inhibiting IGFBP-2 synthesis, inactivating IGFBP-2, or by increasing the breakdown or clearance of IGFBP-2. These three approaches and methods for achieving them are detailed below.

Inhibiting the synthesis is done in cells that are actively synthesizing IGFBP-2. 30 IGFBP-2 may be synthesized by the IGFBP-2 dependent cell, where the IGFBP-2 would be acting in an autocrine manner on the same cell. Alternatively, IGFBP-2 synthesis may be by a second cell distinct from the IGFBP-2 dependent cell in an

endocrine manner. Strategies for inhibiting the synthesis of IGFBP-2 can target transcription of the gene encoding IGFBP-2, target IGFBP-2 messenger RNA, target translation of the IGFBP-2 message, or target secretion of IGFBP-2.

5 Several methods are contemplated for inhibiting IGFBP-2 transcription. The promoter for IGFBP-2 has been isolated and the inventors describe assays for determining the activity of this promoter in a given cell. Methods for inhibiting transcription include synthesis of antisense RNA complementary to the IGFBP-2 nucleic acid sequence. Antisense RNA can interact with genomic DNA, forming
10 heteroduplexes that inhibit transcription of the DNA sequence. Alternative methods could include targeting transcription factors or other accessory factors involved in transcription. Methods for introducing genetic constructs into IGFBP-2 producing cells are described, as are methods for determining IGFBP-2 expression and screens for candidate compounds that affect IGFBP-2 transcription.

15

An alternative approach for inhibiting IGFBP-2 is to target the IGFBP-2 messenger RNA. Inhibition of IGFBP-2 synthesis could either be brought about by decreasing the amount of IGFBP-2 mRNA or inhibiting its translation into IGFBP-2 polypeptide. Methods for achieving this include producing antisense IGFBP-2 RNA
20 that forms a duplex with IGFBP-2 mRNA or producing IGFBP-2 specific ribozymes. Again, methods for introducing genetic constructs into IGFBP-2 producing cells are described, as are methods for determining IGFBP-2 expression and screens for candidate compounds that effect IGFBP-2 translation.

25

IGFBP-2 is a secreted protein and methods for inhibiting its secretion would decrease IGFBP-2 activity. Cells secrete proteins through a secretory pathway, where proteins destined for secretion are targeted to the endoplasmic reticulum. These proteins then pass on to the golgi apparatus and then to the plasma membrane where they are ultimately released. One general method of inhibiting IGFBP-2 secretion is
30 based on producing polypeptides that would interact with IGFBP-2 inside the cell and interfere with its secretion. Such polypeptides include antibodies with specificity to IGFBP-2, with single chain antibodies described as one approach. Alternatively,

several polypeptides are known to interact with IGFBP-2, including IGF-I, IGF-II, and IGFBP receptors. Cosynthesis of such polypeptides, or fragments or fusion proteins containing such polypeptides, could interfere with the secretion of IGFBP-2.

5 Polypeptides such as those described above can also be used to bind and reduce IGFBP-2 activity after it has been secreted from the cell synthesizing it. Methods for using polypeptides that interact with IGFBP-2, including IGF-I, IGF-II, and IGFBP receptors, or fragments or fusion proteins containing such polypeptides are anticipated. In addition, antibodies specific for IGFBP-2 can be used to bind and inactivate IGFBP-
10 2. Such antibodies can be monoclonal or polyclonal as well as human or humanized antibodies. Polypeptides such as these that bind IGFBP-2 can lead to its inactivation, increased clearance rate or shorter half-life, all of which will decrease overall IGFBP-2 activity.

15 A final method contemplated for reducing IGFBP-2 activity is to effect post-translational modifications that affect its activity. Post-translational modifications that can be targeted include phosphorylation, proteolysis and glycosylation, as well as polypeptides involved in these modifications, including kinases, phosphatases and proteases.

20
IGFBP-2 is a major prostatic binding protein and is dramatically elevated in serum of all patients with prostate cancer, but not in non-cancerous benign prostatic hyperplasia; the degree of elevation is directly related to the severity of the cancer. This suggests that IGFBP-2 may be involved in the development and/or progression of
25 prostate cancer. However, the role of IGFBP-2 in prostatic carcinogenesis was previously not known. An investigation was undertaken to determine the effects of IGFBP-2 and IGFBP-2 antibody on the growth of androgen-sensitive LNCap and androgen-independent DU145 prostate cancer cells in the presence or absence of IGF-I. The inventors' results demonstrate that the growth of these cell lines was both
30 significantly potentiated by IGFBP-2 and significantly retarded by either IGFBP-2 antibody or suppression of endogenous IGFBP-2 in transfection studies. In addition to providing a method for producing therapeutic compounds, these findings have

profound clinical implications in the diagnosis and treatment of prostate cancer. IGFBP-2 antibody and endogenous suppression of IGFBP-2 can inhibit the growth of prostate tumors *in vitro*, then: (1) circulatory levels of IGFBP-2 can serve as a biomarker for prostatic carcinoma, and (2) suppression of IGFBP-2 by various modes 5 of action can be used as a novel strategy for treating prostate cancer.

1. INSULIN-LIKE GROWTH FACTOR AND BINDING PROTEINS

Insulin-like Growth Factors (IGFs) are low molecular weight polypeptide hormones with structural homology to proinsulin. Two different IGFs are known, 10 namely IGF-I and IGF-II, which are mitogenic *in vitro* for a wide variety of cells in tissue culture. A general review of the Insulin-like Growth Factors is given in Jones and Clemons (1995), incorporated herein by reference. Both IGFs stimulate *in vitro* the growth of various tissues and in particular they induce collagen synthesis. IGF-I mediates the growth promoting effect of growth hormone in chondrogenesis and bone 15 formation and is therefore essential for normal growth of an organism. This is demonstrated by the fact that pygmies and toy poodles are deficient in IGF-I, but have normal growth hormone level in their serum. IGF-II is believed to play a key role in fetal development and nerve growth.

20 In addition to their primary effect on skeletal tissue, IGF-I and IGF-II also exhibit growth-stimulating functions on other tissues. Wound fibroblasts are known to produce IGFs that are effective in stimulating fibroblasts to grow and synthesize collagen, a structural protein normally required for wound healing. Vascularization of the wound tissue also is induced. Further, it has also been found that IGFs have an 25 erythropoietin-like activity in that they induce hematopoiesis.

Recent studies also have demonstrated that IGFs produced by certain cancer 30 cells, e.g., breast and kidney cancer cells, auto-stimulate the proliferation of cancer cells and the vascular and fibrous tissues required to support the growth of cancer tissues.

In addition to this, both IGFs show a spectrum of metabolic activities similar to those of insulin, in that they stimulate, in particular, the transport and metabolism of glucose. The biological effects of IGFs and insulin are mediated through their binding to specific receptors. In particular, both IGFs have the ability to bind to the insulin receptor with approximately 100-fold lower affinity than does insulin.

Both IGFs have a concentration in blood approximately a hundred-fold higher than that of insulin. Hypoglycemia is prevented by a regulatory mechanism that involves carrier proteins present in blood and able to form complexes with IGFs. Thus, 10 IGFs circulate in the blood in the form of a complex that has no insulin-like activity. Through their association with carrier protein (hereinafter referred to as IGF-binding proteins or IGFBPs), binding of IGFs to cell surface receptors is inhibited. It also has been demonstrated that another function of the IGF-binding proteins is to increase the short half-life of IGFs, which are subjected to rapid proteolytic degradation when 15 present in the free form in blood.

A. INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

In the circulation, in other body fluids, and in media conditioned by cultured cells, the somatomedins (IGF-I and IGF-II) are bound to specific high-affinity carrier 20 proteins that have been implicated as modulators of IGF actions. The history of IGF binding proteins (BPs) dates back to 1984 when the existence of specific somatomedin carrier proteins in serum was first shown (Hintz, 1984). Four distinct IGFBPs have now been cloned and sequenced, and in addition, several other, not yet thoroughly characterized, BP species have been identified in various tissues (Baxter and Martin, 25 1989; Roghani *et al.*, 1989; Bautista *et al.*, 1990). On the basis of the sequences it became evident that many of the previously recognized BPs, known by different names, were in fact the same, falling into a defined number of classes of cloned BPs. To clarify the status of these BPs, the Workshop on IGF Binding Proteins, held in Vancouver, Canada, June 1989, proposed the names IGFBP-1, IGFBP-2, and IGFBP- 30 3 for the binding proteins with defined sequences (Ballard *et al.*, 1989). The consensus at the Workshop was that other incompletely characterized IGFBPs be referred to by

size and origin until sequenced. Since that time, several other IGFBPs, namely IGFBP-4, IGFBP-5 and IGFBP-6 have been sequenced, as described below.

Various review articles report that despite increasing interest in IGFBPs in
5 recent years, their functions are still poorly understood (Baxter, 1988; Jones and Clemons, 1995). Baxter points to some evidence that association with BPs may not always inhibit the activity of the IGFs and that cell types producing the BPs might be able to enhance their IGF responsiveness in an autocrine fashion. Examples cited are that (1) some high molecular weight complexes from human plasma retain biological
10 activity in rat adipocyte assays for insulin-like activity; (2) cultured human fibroblasts secrete a BP of 35 kD that increases cell IGF binding; and, (3) a pure preparation of amniotic fluid BP significantly potentiates the effect of IGF-I in stimulating DNA synthesis in porcine smooth muscle cells and fibroblasts from various species. Furthermore, it has been shown that IGFBP-3 blocks the hypoglycemic action of IGF-I
15 when administered subcutaneously together with the IGF-I in a 1:1 ratio (Spencer *et al.*, 1991).

Another view is that IGFBPs are produced locally in all tissues to concentrate
20 locally produced IGF-I near cells requiring the IGF-I, reducing the active role of IGF-I bound to BPs and IGF-I circulating in the blood (Isaksson *et al.*, 1987). It has been reported, for example, that IGF-I is produced locally in bone by GH (Nilsson *et al.*, 1986), and GH receptors have been found on chondrocytes (Nilsson *et al.*, 1989).

Four major functions have been proposed for IGFBPs in regulating the
25 activities of IGFs. These are 1) to act as transport proteins in plasma and to modulate the location and movement of bound IGFs; 2) to modulate the half-life and clearance rates of IGFs; 3) to provide a means of tissue- and cell-type specific localization of IGFs; and 4) to directly modulate interactions between IGFs and IGF receptors and the resulting effects of receptor binding. However, evidence also exists for more direct
30 biologic functions of IGFBPs, including direct effects of IGFBPs on cell function in the absence of IGFs and specific cellular receptors for IGFBPs distinct from IGF receptors.

Amniotic fluid was the first source from which IGFBP-1 was detected (Chochinov *et al.*, 1977). The protein has been purified also from tissue extract of fetal and maternal placenta and named placental protein (Kiostinen *et al.*, 1986). The 5 mature protein contains 234 amino acids, predicting a molecular mass of 25.3 kD (Lee *et al.*, 1988; WO 89/09792. IGFBP-1 migrates on SDS-PAGE at 28-35 kD depending on the stage of reduction. IGFBP-1 is a minor binding protein in serum and contains the unsaturated serum IGF-binding sites. Serum levels are inversely dependent on insulin and have a marked diurnal variation, the levels being highest early in the 10 morning. These levels increase in pregnancy up to several hundred ug/L, and amniotic fluid levels are up to 1000-fold higher than those in serum.

Carrier proteins of the IGFBP-2 class have been isolated from human fetal liver and rat and bovine cell lines (Binkert *et al.*, 1989; Rosenfeld *et al.*, 1990). In humans, 15 the mature form contains 289 amino acids and has an apparent molecular mass of 31-40 kD, depending on the stage of reduction on SDS-PAGE. In humans high IGFBP-2 levels have been found in the cerebrospinal fluid. The abundance of this protein in fetal tissue suggests that it has a role in regulating development. IGFBP-2 preferentially binds IGF-II. The nucleic acid sequence of the cDNA encoding human IGFBP-2 is 20 shown as SEQ ID NO:1 and the deduced amino acid sequence of human IGFBP-2 is shown as SEQ ID NO:2.

The majority of serum IGFs are bound to a BP composed of two parts forming a complex of molecular mass 125-150 kD. IGFBP-3 is the IGF binding subunit (β -subunit) in this complex (Baxter and Martin, 1989). It is an acid-stable glycoprotein appearing on SDS-PAGE as a major and minor band, corresponding to molecular weights of 53 kD and 47 kD, respectively. The other components in the complex are the acid-labile, non-IGF-binding subunit (α -subunit) with a molecular mass of 84-86 kD (Baxter, WO 90/0569), and IGF-I or IGF-II (γ -subunit). Sequencing of the cloned 25 cDNA for IGFBP-3 (previously known as IGFBP-53) predicts a molecular mass of 30 28.7 kD for the non-glycosylated protein and reveals that IGFBP-3 shares 33%

sequence identity with IGFBP-1 (Wood *et al.*, 1988; WO 89/09268 published Oct. 5, 1989).

A 25-kD IGFBP-4 has been isolated from cultured human osteoblast-like TE89
5 osteosarcoma cell conditioned media and sequenced (Mohan *et al.*, 1989). A similar, if not identical, IGFBP was isolated from human prostatic carcinoma cells and sequenced (Perkel *et al.*, 1990). Another similar IGFBP was identified in adult rat serum (Shimonaka *et al.*, 1989).

10 IGFBP-5 is a 252 amino acid protein that is highly conserved between species (White *et al.*, 1996). Among the IGFBPs produced IGFBP-5 is unique with regard to its marked affinity to matrix of normal bone cells, its short half-life when released, and its stimulatory effects on DNA synthesis (Schmid *et al.*, 1995). Extracellular matrix binding of IGFBP-5 leads to a decrease in its affinity for insulin-like growth factor-I
15 (IGF-I), which allows IGF-I to better equilibrate with IGF receptors (Parker *et al.*, 1998).

20 In a myoblast system, IGFBP-5 stimulated all aspects of the myogenic response to IGF-I: the later rise in myogenin mRNA, the elevation of creatine kinase activity, and the fusion of myoblasts into myotubes. In contrast, when IGFBP-5 was added in the presence of IGF-II, it inhibited both proliferation and differentiation (Ewton *et al.* 1998). This was seen in other studies, where IGFBP-5 inhibits muscle differentiation, implying a role for IGFBP-5 in regulating IGF action during myogenic development *in vivo* (James *et al.*, 1996).

25

IGFBP-6 has only recently been isolated and cloned. It is expressed during mouse development in a number of tissues, including brain (Putzer *et al.*, 1998). The effects of IGFBP-6 on neuroblastoma cell growth has been studied. (Grellier *et al.*, 1998; Babajko *et al.*, 1997) These findings suggest that IGFBP-6 contributes as an
30 autocrine inhibitor in the regulation of growth by the IGF system in these neuroblastoma cells

In other studies on IGFBP-6 actions, IGFBP-6 completely inhibited IGF-II-induced [³H]thymidine incorporation in MC3T3-E1 mouse osteoblast cells, while it had only minimal inhibitory effects on IGF-I-induced [³H]thymidine incorporation. This differential effect is associated with the fact that IGFBP-6 has greater affinity for IGF-II than IGF-I (Srinivasan *et al.*, 1996).

The levels of IGFBP in adult serum have been found to reflect the growth hormone (GH) status of individuals who are either GH-deficient or acromegalic. Thus, high levels of IGFBP-3 correlate with high levels of GH (Martin and Baxter, 1985).

10 Under normal conditions about 95-98% of the IGF-I in human plasma is bound to the IGFBPs. Studies on size-fractionated human serum, subjected to IGF-I RIA after extraction of each fraction to remove binding activity, have indicated that 72% of the endogenous peptide is associated with the 150-kD fraction and 25% with the 50-kD fraction (Daughaday *et al.*, 1982).

15

B. IGFBP-2 ACTIVITY

The specific actions of IGFBP-2 and IGFs are unclear. Purified IGFBP-2 has been shown to inhibit [³H] thymidine incorporation into DNA in two different cell systems (Rechler and Nissley, 1990; Knauer and Smith, 1980). Inhibition of DNA synthesis was also seen using purified human IGFBP-2 in a human lung carcinoma line (Reeve *et al.*, 1993). One explanation for the observed inhibitory actions is that IGFBP-2 inhibited binding of IGFs to IGF cell surface receptors (Ross *et al.*, 1989). However, IGFBP-2 has been shown to be a weak potentiator of IGF action as well. IGFBP-2 was shown to enhance the effect of IGF-I in microvascular endothelial cells 25 in serum-free medium (Bar *et al.*, 1989). Also, in aortic smooth muscle cells, IGFBP-2 was shown to potentiate the actions of IGF (Bourner *et al.*, 1992).

Experimental results describing actions of of IGFBP-2, and IGFBPs in general, do have to be interpreted with caution, since the extent of post-translational 30 modification of the various IGFBPs is still under investigation. IGFBPs are known to be modified by phosphorylation (Jones *et al.*, 1991 and 1992; Mukku and Chu, 1990) and glycosylation. Proteolysis by calcium dependent serine proteases in serum or other

extracellular locations also occurs and can alter biologic activity. Finally, different target cells will have give different and often times contradictory results with respect to evaluating the biologic properties of a given IGFBP. This can most certainly be due to the identification of specific receptors or binding sites for IGFBPs.

5

As used herein, "IGFBP" refers to a protein that binds IGFs in the circulation, in other body fluids, and in media conditioned by cultured cells, as defined in the Workshop on IGF Binding Proteins held in Vancouver, Canada in June 1989 discussed above and reported in Ballard *et al.* (1989). This term includes IGFBP-1, IGFBP-2, 10 IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, and other as yet unidentified IGFBPs that have the characteristics common to all the known IGF binding proteins. The term includes animal equivalents to human IGFBPs as well as human IGFBPs, for example, the bovine, ovine, murine, porcine, and equine species. It may be from any source, whether natural, synthetic, or recombinant, provided that it will bind to the appropriate 15 binding domain of IGFs.

The term "IGFBP-2 dependent cancer cell" or "IGFBP-2 dependent cell" refers to cells whose biologic activity is modulated by IGFBP-2. Specific biologic properties or cell functions contemplated in this application include altering proliferation, 20 metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion. Other described functions of IGFBP-2 may include modulating chemotaxis, chemokinesis, immune response, and amino acid and glucose uptake. These responses may be mediated directly by IGFBP-2 or by IGFBP-2's modulation of IGF function as described above. Cells other than 25 cancer that are possibly affected by IGFBP-2 include fibroblasts, smooth muscle cells, chondrocytes, osteoblasts, keratinocytes, skeletal muscle cells, neuronal cells, mammary epithelial cells, mesangial cells, thyroid follicular cells, erythroid progenitor cells, thymic epithelium, oocytes, granulosa cells, spermatogonia, Sertoli cells and prostate cells.

30

2. ANTIBODIES TO IGFBP-2 POLYPEPTIDES AND PEPTIDES

Another embodiment of the present invention is a monoclonal antibody and more preferably a human monoclonal antibody immunoreactive with the peptide sequence designated herein as SEQ ID NO:2. It is understood that antibodies can be used for 5 inhibiting an IGFBP-2 dependent cancer cell by binding or modulating IGFBP-2. It is also understood that this antibody is useful for screening samples from human patients for the purpose of detecting IGFBP-2 present in the samples. The antibody also may be useful in the screening of expressed DNA segments or peptides and proteins for the discovery of related antigenic sequences. In addition, the antibody may be useful in 10 passive immunotherapy for cancer. All such uses of the said antibody and any antigens or epitopic sequences so discovered fall within the scope of the present invention.

A. ANTIBODY GENERATION

In certain embodiments, the present invention provides antibodies that bind 15 with high specificity to the IGFBP-2 polypeptides provided herein. Thus, antibodies that bind to the protein products of the isolated nucleic acid sequences of SEQ ID NO:1 are provided. As detailed above, in addition to antibodies generated against the full length proteins, antibodies also may be generated in response to smaller constructs comprising epitopic core regions, including wild-type and mutant epitopes.

20

As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE. Generally, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting.

25

Monoclonal antibodies (MAbs) are recognized to have certain advantages, e.g., reproducibility and large-scale production, and their use is generally preferred. The invention thus provides monoclonal antibodies of the human, murine, monkey, rat, hamster, rabbit and even chicken origin. Due to the ease of preparation and ready 30 availability of reagents, murine monoclonal antibodies will often be preferred.

However, "humanized" antibodies are also contemplated, as are chimeric antibodies from mouse, rat, or other species, bearing human constant and/or variable region domains, bispecific antibodies, recombinant and engineered antibodies and fragments thereof. Methods for the development of antibodies that are 5 "custom-tailored" to the patient's disease are likewise known and such custom-tailored antibodies are also contemplated.

The term "antibody" is used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, F(ab')₂, 10 single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, e.g., Harlow and Lane, "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

15

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic IGFBP-2 polypeptide composition in accordance with the present invention and collecting 20 antisera from that immunized animal.

A wide range of animal species can be used for the production of antisera. Typically the animal used for production of antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of 25 rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. 30 Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin also can be used as carriers. Means for conjugating a

polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

5 As also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Suitable molecule adjuvants include all acceptable immunostimulatory compounds, such as cytokines, toxins or synthetic compositions.

10 Adjuvants that may be used include IL-1, IL-2, IL-4, IL-7, IL-12, γ -interferon, GM-CSF, BCG, aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion also is contemplated. MHC antigens may even be used. Exemplary, often preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

15 20 In addition to adjuvants, it may be desirable to coadminister biologic response modifiers (BRM), which have been shown to upregulate T cell immunity or downregulate suppressor cell activity. Such BRMs include, but are not limited to, Cimetidine (CIM; 1200 mg/d) (Smith/Kline, PA); low-dose Cyclophosphamide (CYP; 300 mg/m²) (Johnson/ Mead, NJ), cytokines such as γ -interferon, IL-2, or IL-12 or 25 genes encoding proteins involved in immune helper functions, such as B-7.

25 The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The

production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization..

A second, booster injection also may be given. The process of boosting and
5 titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

For production of rabbit polyclonal antibodies, the animal can be bled through
10 an ear vein or alternatively by cardiac puncture. The removed blood is allowed to coagulate and then centrifuged to separate serum components from whole cells and blood clots. The serum may be used as is for various applications or else the desired antibody fraction may be purified by well-known methods, such as affinity chromatography using another antibody, a peptide bound to a solid matrix, or by using,
15 e.g., protein A or protein G chromatography.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected
20 immunogen composition, e.g., a purified or partially purified IGFBP-2 polypeptide, peptide or domain, be it a wild-type or mutant composition. The immunizing composition is administered in a manner effective to stimulate antibody producing cells.

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep or frog cells also is possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred as this is most
25 30 routinely used and generally gives a higher percentage of stable fusions.

The animals are injected with antigen, generally as described above. The antigen may be coupled to carrier molecules such as keyhole limpet hemocyanin if necessary. The antigen would typically be mixed with adjuvant, such as Freund's complete or incomplete adjuvant. Booster injections with the same antigen would 5 occur at approximately two-week intervals.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or 10 lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible.

15 Often, a panel of animals will have been immunized and the spleen of an animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

20 The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective 25 media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, 30 NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210;

and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed 5 P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

10 Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described 15 by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods also is appropriate (Goding pp. 71-74, 1986).

20 Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8} . However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that 25 blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with 30 hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, e.g., hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate 5 this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific 10 hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the 15 like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in 20 two basic ways. First, a sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion (e.g., a syngeneic mouse). Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. The injected animal develops tumors 25 secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. Second, the individual cell lines could be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations.

30

MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or

affinity chromatography. Fragments of the monoclonal antibodies of the invention can be obtained from the monoclonal antibodies so produced by methods which include digestion with enzymes, such as pepsin or papain, and/or by cleavage of disulfide bonds by chemical reduction. Alternatively, monoclonal antibody fragments 5 encompassed by the present invention can be synthesized using an automated peptide synthesizer.

It also is contemplated that a molecular cloning approach may be used to generate monoclonals. For this, combinatorial immunoglobulin phagemid libraries are 10 prepared from RNA isolated from the spleen of the immunized animal, and phagemids expressing appropriate antibodies are selected by panning using cells expressing the antigen and control cells. The advantages of this approach over conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H 15 and L chain combination which further increases the chance of finding appropriate antibodies.

Humanized monoclonal antibodies are antibodies of animal origin that have been modified using genetic engineering techniques to replace constant region and/or 20 variable region framework sequences with human sequences, while retaining the original antigen specificity. Such antibodies are commonly derived from rodent antibodies with specificity against human antigens. Such antibodies are generally useful for *in vivo* therapeutic applications. This strategy reduces the host response to the foreign antibody and allows selection of the human effector functions.

25

The techniques for producing humanized immunoglobulins are well known to those of skill in the art. For example US Patent No. 5,693,762 discloses methods for producing, and compositions of, humanized immunoglobulins having one or more complementarity determining regions (CDR's). When combined into an intact 30 antibody, the humanized immunoglobulins are substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin to the antigen, such as a protein or other compound containing an epitope.

Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer, or by expression of full-length gene or of gene fragments in *E. coli*.

5

B. IGFBP-2 ANTIGENIC SEQUENCES

As another way of effecting modulation of IGFBP-2 in a subject, peptides corresponding to one or more antigenic determinants of the IGFBP-2 polypeptides of the present invention also can be prepared so that an immune response against IGFBP-10 2 is raised. Thus, it is contemplated that vaccination with an IGFBP-2 peptide or polypeptide may generate an autoimmune response in an immunized animal such that autoantibodies that specifically recognize the animal's endogenous IGFBP-2 protein. This vaccination technology is shown in U.S. Patent Nos. 6,027,727; 5,785,970, and 5,609,870, which are hereby incorporated by reference.

15

Such peptides should generally be at least five or six amino acid residues in length and will preferably be about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or about 30 amino acid residues in length, and may contain up to about 35-50 residues. For example, these peptides may comprise IGFBP-2 amino acid sequence, such as 20 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, and 50 or more contiguous amino acids from SEQ ID NO:2. Synthetic peptides will generally be about 35 residues long, which is the approximate upper length limit of automated peptide synthesis machines, such as those available from Applied Biosystems (Foster City, CA). Longer peptides also may be prepared, e.g., by 25 recombinant means.

U.S. Patent 4,554,101, (Hopp) incorporated herein by reference, teaches the identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed in Hopp, one of skill in the art 30 would be able to identify epitopes from within an amino acid sequence such as the IGFBP-2 sequence disclosed herein in SEQ ID NO: 2. A peptide with the sequence

LFYNEQQEARGVHTQRMQ (SEQ ID NO:3) has been used to generate antibodies against human IGFBP-2.

Numerous scientific publications have also been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou & Fasman, 1974a,b; 1978a,b, 1979). Any of these may be used, if desired, to supplement the teachings of Hopp in U.S. Patent 4,554,101.

Moreover, computer programs are currently available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson & Wolf, 1988; Wolf *et al.*, 1988), the program PepPlot® (Brutlag *et al.*, 1990; Weinberger *et al.*, 1985), and other new programs for protein tertiary structure prediction (Fetrow & Bryant, 1993). Another commercially available software program capable of carrying out such analyses is MacVector (IBI, New Haven, CT).

In further embodiments, major antigenic determinants of a IGFBP-2 polypeptide may be identified by an empirical approach in which portions of the gene encoding the IGFBP-2 polypeptide are expressed in a recombinant host, and the resulting proteins tested for their ability to elicit an immune response. For example, PCR™ can be used to prepare a range of peptides lacking successively longer fragments of the C-terminus of the protein. The immunoactivity of each of these peptides is determined to identify those fragments or domains of the polypeptide that are immunodominant. Further studies in which only a small number of amino acids are removed at each iteration then allows the location of the antigenic determinants of the polypeptide to be more precisely determined.

Another method for determining the major antigenic determinants of a polypeptide is the SPOTs™ system (Genosys Biotechnologies, Inc., The Woodlands, TX). In this method, overlapping peptides are synthesized on a cellulose membrane, which following synthesis and deprotection, is screened using a polyclonal or

monoclonal antibody. The antigenic determinants of the peptides which are initially identified can be further localized by performing subsequent syntheses of smaller peptides with larger overlaps, and by eventually replacing individual amino acids at each position along the immunoreactive peptide.

5

Once one or more such analyses are completed, polypeptides are prepared that contain at least the essential features of one or more antigenic determinants. The peptides are then employed in the generation of antisera against the polypeptide. Minigenes or gene fusions encoding these determinants also can be constructed and 10 inserted into expression vectors by standard methods, for example, using PCR™ cloning methodology.

The use of such small peptides for antibody generation or vaccination typically requires conjugation of the peptide to an immunogenic carrier protein, such as hepatitis 15 B surface antigen, keyhole limpet hemocyanin or bovine serum albumin, or other adjuvants discussed above (adjuvenated peptide). Alum is an adjuvant that has proven sufficiently non-toxic for use in humans. Methods for performing this conjugation are well known in the art. Other immunopotentiating compounds are also contemplated for use with the compositions of the invention such as polysaccharides, including 20 chitosan, which is described in U.S. Patent No. 5,980,912, hereby incorporated by reference. Multiple (more than one) IGFBP-2 epitopes may be crosslinked to one another (e.g., polymerized). Alternatively, a nucleic acid sequence encoding an IGFBP-2 peptide or polypeptide may be combined with a nucleic acid sequence that heightens the immune response. Such fusion proteins may comprise part or all of a 25 foreign (non-self) protein such as bacterial sequences, for example.

Antibody titers effective to achieve a response against endogenous IGFBP-2 will vary with the species of the vaccinated animal, as well as with the sequence of the administered peptide. However, effective titers may be readily determined, for 30 example, by testing a panel of animals with varying doses of the specific antigen and measuring the induced titers of autoantibodies (or anti-self antibodies) by known

techniques, such as ELISA assays, and then correlating the titers with IGFBP-2-related cancer characteristics, *e.g.*, tumor growth or size.

One of ordinary skill would know various assays to determine whether an immune response against IGFBP-2 was generated. The phrase "immune response" includes both cellular and humoral immune responses. Various B lymphocyte and T lymphocyte assays are well known, such as ELISAs, cytotoxic T lymphocyte (CTL) assays, such as chromium release assays, proliferation assays using peripheral blood lymphocytes (PBL), tetramer assays, and cytokine production assays. See Benjamini et al., 1991, hereby incorporated by reference.

C. LINKERS/COUPLING AGENTS

If desired, dimers or multimers of IGFBP-2 peptides, or an IGFBP-2 peptide and a non-self, non-IGFBP-2 peptide or adjuvant may be joined *via* a biologically-releasable bond, such as a selectively-cleavable linker or amino acid sequence. For example, peptide linkers that include a cleavage site for an enzyme preferentially located or active within a tumor environment are contemplated. Exemplary forms of such peptide linkers are those that are cleaved by urokinase, plasmin, thrombin, Factor IXa, Factor Xa, or a metallaproteinase, such as collagenase, gelatinase, or stromelysin. Alternatively, the linkers and coupling agents described herein may be employed to join an anti-IGFBP-2 antibody with a therapeutic or preventative agent, such as a toxin.

Amino acids such as selectively-cleavable linkers, synthetic linkers, or other amino acid sequences may be used to separate an IGFBP-2 peptide from another peptide or adjuvant, or anti-IGFBP-2 antibodies from a therapeutic compound.

Additionally, while numerous types of disulfide-bond containing linkers are known that can successfully be employed to conjugate the toxin moiety with the targeting agent, certain linkers will generally be preferred over other linkers, based on differing pharmacologic characteristics and capabilities. For example, linkers that contain a disulfide bond that is sterically "hindered" are to be preferred, due to their greater stability *in vivo*, thus preventing release of the toxin moiety prior to binding at

the site of action. Furthermore, while certain advantages in accordance with the invention will be realized through the use of any of a number of toxin moieties, the inventors have found that the use of ricin A chain, and even more preferably deglycosylated A chain, will provide particular benefits.

5

i) Biochemical cross-linkers

It can be considered as a general guideline that any biochemical cross-linker that is appropriate for use in an immunotoxin will also be of use in the present context, and additional linkers may also be considered.

10

Cross-linking reagents are used to form molecular bridges that tie together functional groups of two different molecules, *e.g.*, a stabilizing and coagulating agent. To link two different proteins in a step-wise manner, hetero-bifunctional cross-linkers can be used that eliminate unwanted homopolymer formation.

TABLE 1
HETERO-BIFUNCTIONAL CROSS-LINKERS

linker	Reactive Toward	Advantages and Applications	Spacer Arm Length\after cross-linking
SMPT	Primary amines Sulphydryls	· Greater stability	11.2 Å
SPDP	Primary amines Sulphydryls	· Thiolation · Cleavable cross-linking	6.8 Å
LC-SPDP	Primary amines Sulphydryls	· Extended spacer arm	15.6 Å
Sulfo-LC-SPDP	Primary amines Sulphydryls	· Extended spacer arm · Water-soluble	15.6 Å
SMCC	Primary amines Sulphydryls	· Stable maleimide reactive group · Enzyme-antibody conjugation · Hapten-carrier protein conjugation	11.6 Å
Sulfo-SMCC	Primary amines Sulphydryls	· Stable maleimide reactive group · Water-soluble · Enzyme-antibody conjugation	11.6 Å
MBS	Primary amines Sulphydryls	· Enzyme-antibody conjugation · Hapten-carrier protein conjugation	9.9 Å
Sulfo-MBS	Primary amines Sulphydryls	· Water-soluble	9.9 Å
SIAB	Primary amines Sulphydryls	· Enzyme-antibody conjugation	10.6 Å
Sulfo-SIAB	Primary amines Sulphydryls	· Water-soluble	10.6 Å
SMPB	Primary amines Sulphydryls	· Extended spacer arm · Enzyme-antibody conjugation	14.5 Å
Sulfo-SMPB	Primary amines Sulphydryls	· Extended spacer arm · Water-soluble	14.5 Å
EDC/Sulfo-NHS	Primary amines Carboxyl groups	· Hapten-Carrier conjugation	0
ABH	Carbohydrates Nonselective	· Reacts with sugar groups	11.9 Å

An exemplary hetero-bifunctional cross-linker contains two reactive groups: one reacting with primary amine group (e.g., N-hydroxy succinimide) and the other reacting with a thiol group (e.g., pyridyl disulfide, maleimides, halogens, etc.). Through the primary amine reactive group, the cross-linker may react with the lysine residue(s) of one protein (e.g., the selected antibody or fragment) and through the thiol reactive group, the cross-linker, already tied up to the first protein, reacts with the cysteine residue (free sulfhydryl group) of the other protein (e.g., the selective agent).

It can therefore be seen that a targeted peptide composition will generally have, or be derivatized to have, a functional group available for cross-linking purposes. This requirement is not considered to be limiting in that a wide variety of groups can be used in this manner. For example, primary or secondary amine groups, hydrazide or hydrazine groups, carboxyl alcohol, phosphate, or alkylating groups may be used for binding or cross-linking. For a general overview of linking technology, one may wish to refer to Ghose & Blair (1987).

The spacer arm between the two reactive groups of a cross-linkers may have various length and chemical compositions. A longer spacer arm allows a better flexibility of the conjugate components while some particular components in the bridge (e.g., benzene group) may lend extra stability to the reactive group or an increased resistance of the chemical link to the action of various aspects (e.g., disulfide bond resistant to reducing agents). The use of peptide spacers, such as L-Leu-L-Ala-L-Leu-L-Ala, is also contemplated.

It is preferred that a cross-linker having reasonable stability in blood will be employed. Numerous types of disulfide-bond containing linkers are known that can be successfully employed to conjugate targeting and therapeutic/preventative agents. Linkers that contain a disulfide bond that is sterically hindered may prove to give greater stability *in vivo*, preventing release of the targeting peptide prior to reaching the site of action. These linkers are thus one group of linking agents.

Another cross-linking reagents for use in immunotoxins is SMPT, which is a bifunctional cross-linker containing a disulfide bond that is "sterically hindered" by an adjacent benzene ring and methyl groups. It is believed that stearic hindrance of the disulfide bond serves a function of protecting the bond from attack by thiolate anions
5 such as glutathione which can be present in tissues and blood, and thereby help in preventing decoupling of the conjugate prior to the delivery of the attached agent to the tumor site. It is contemplated that the SMPT agent may also be used in connection with the bispecific coagulating ligands of this invention.

10 The SMPT cross-linking reagent, as with many other known cross-linking reagents, lends the ability to cross-link functional groups such as the SH of cysteine or primary amines (*e.g.*, the epsilon amino group of lysine). Another possible type of cross-linker includes the hetero-bifunctional photoreactive phenylazides containing a cleavable disulfide bond such as sulfosuccinimidyl-2-(*p*-azido salicylamido) ethyl-1,3'-dithiopropionate. The N-hydroxy-succinimidyl group reacts with primary amino groups and the phenylazide (upon photolysis) reacts non-selectively with any amino acid residue.
15

20 In addition to hindered cross-linkers, non-hindered linkers also can be employed in accordance herewith. Other useful cross-linkers, not considered to contain or generate a protected disulfide, include SATA, SPD_P and 2-iminothiolane (Wawrzynczak & Thorpe, 1988). The use of such cross-linkers is well understood in the art.

25 Once conjugated, the peptide generally will be purified to separate the conjugate from unconjugated targeting agents or coagulants and from other contaminants. A large number of purification techniques are available for use in providing conjugates of a sufficient degree of purity to render them clinically useful. Purification methods based upon size separation, such as gel filtration, gel permeation
30 or high performance liquid chromatography, will generally be of most use. Other chromatographic techniques, such as Blue-Sepharose separation, may also be used.

In addition to chemical conjugation, an IGFBP-2 polypeptide, peptide, or antibody may be modified at the protein level. Included within the scope of the invention are IgA protein fragments or other derivatives or analogs that are differentially modified during or after translation, for example by glycosylation, 5 acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, and proteolytic cleavage. Any number of chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, farnesylation, oxidation, reduction; metabolic synthesis in the 10 presence of tunicamycin.

3. IGFBP-2 NUCLEIC ACIDS

A. GENES AND DNA SEGMENTS

Important aspects of the present invention concern isolated DNA segments and 15 recombinant vectors expressing IGFBP-2 antisense or sense, and the creation and use of recombinant host cells through the application of DNA technology, including such sequences encoding one or more IGFBP-2 polypeptides, or functional equivalents thereof.

20 The present invention concerns DNA segments, isolatable from mammalian and human cells, that are free from total genomic DNA and are capable of expressing IGFBP-2 polypeptide in a recombinant host cell when incorporated into the recombinant host cell.

25 As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding IGFBP-2 refers to a DNA segment that contains coding sequences of IGFBP-2, yet is isolated away from, or purified free from, total mammalian or human genomic DNA. Included within the term "DNA segment", are DNA segments 30 and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified *IGFBP-2* gene refers to a DNA segment including any of the *IGFBP-2* gene coding sequences and, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, cDNA sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides or fusion proteins.

10

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case any *IGFBP-2* gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

20 In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode an *IGFBP-2* polypeptide that includes within its amino acid sequence a contiguous amino acid sequence of human *IGFBP-2* polypeptide, or functional equivalents thereof.

25 Naturally, where the DNA segment or vector encodes an *IGFBP-2* polypeptide, or is intended for use in expressing the *IGFBP-2* polypeptide, the most preferred sequences are those that are essentially as set forth in the contiguous sequence of SEQ ID NO:1.

30 Sequence of an *IGFBP-2* polypeptide will substantially correspond to a contiguous portion of that shown in SEQ ID NO:2, and have relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino

acids shown in SEQ ID NO:2. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein.

Accordingly, sequences that have between about 70% and about 80%; or more 5 preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:2 will be sequences that are "essentially as set forth in SEQ ID NO:2."

10 In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that include within their sequence a contiguous nucleic acid sequence from that shown in SEQ ID NO:1. This definition is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a contiguous portion of that shown in SEQ ID NO:1 and has relatively few codons 15 that are not identical, or functionally equivalent, to the codons of SEQ ID NO:1. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids. See Table 2 below, which lists the codons preferred for use in humans, with the codons listed in decreasing order of 20 preference from left to right in the table (Wada *et al.*, 1990). Codon preferences for other organisms also are well known to those of skill in the art (Wada *et al.*, 1990, included herein in its entirety by reference).

Table 2
Preferred Human DNA Codons

<u>Amino Acids</u>		<u>Codons</u>						
Alanine	Ala	A	GCC	GCT	GC A	GCG		
Cysteine	Cys	C	TGC	TGT				
Aspartic acid	Asp	D	GAC	GAT				
Glutamic acid	Glu	E	GAG	GAA				
Phenylalanine	Phe	F	TTC	TTT				
Glycine	Gly	G	GGC	GGG	GG A	GGT		
Histidine	His	H	CAC	CAT				
Isoleucine	Ile	I	ATC	ATT	AT A			
Lysine	Lys	K	AAG	AAA				
Leucine	Leu	L	CTG	CTC	TTG	CTT	CTA	TTA
Methionine	Met	M	ATG					
Asparagine	Asn	N	AAC	AAT				
Proline	Pro	P	CCC	CCT	CC A	CCG		
Glutamine	Gln	Q	CAG	CAA				
Arginine	Arg	R	CGC	AGG	CG G	AGA	CGA	CGT
Serine	Ser	S	AGC	TCC	TCT	AGT	TCA	TCG
Threonine	Thr	T	ACC	ACA	AC T	ACG		
Valine	Val	V	GTG	GTC	GTT	GTA		
Tryptophan	Trp	W	TGG					
Tyrosine	Tyr	Y	TAC	TAT				

It also will be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the

maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns,
5 which are known to occur within genes.

Excepting intronic or flanking regions, and allowing for the degeneracy of the genetic code, sequences that have between about 70% and about 79%; or more preferably, between about 80% and about 89%; or even more preferably, between
10 about 90% and about 99% of nucleotides that are identical to the nucleotides shown in the sequences of SEQ ID NO:1 will be sequences that are "essentially as set forth in SEQ ID NO:1". Sequences that are essentially the same as those set forth in SEQ ID NO:1 also may be functionally defined as sequences that are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NO:1 under relatively
15 stringent conditions. Suitable relatively stringent hybridization conditions will be well known to those of skill in the art.

Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID
20 NO:1. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid
25 segment of SEQ ID NO:1 under relatively stringent conditions.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning
30 sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of

preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared that include a short contiguous stretch identical to or complementary to the sequence shown in SEQ ID NO:1, such as about 14 nucleotides, and that are up to about 10,000 or about 5,000 base pairs in length, with segments of
5 about 3,000 being preferred in certain cases. DNA segments with total lengths of about 1,000, about 500, about 200, about 100 and about 50 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

It will be readily understood that "intermediate lengths", in these contexts,
10 means any length between the quoted ranges, such as 14, 15, 16, 17, 18, 19, 20, etc.; 21, 22, 23, etc.; 30, 31, 32, etc.; 50, 51, 52, 53, etc.; 100, 101, 102, 103, etc.; 150, 151, 152, 153, etc.; including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000 ranges, up to and including sequences of about 12,001, 12,002, 13,001, 13,002 and the like.
15

The various probes and primers designed around the nucleotide sequences of the present invention may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, etc., an algorithm defining all primers can be proposed:

20 n to n + y

where n is an integer from 1 to the last number of the sequence and y is the length of the primer minus one, where n + y does not exceed the last number of the sequence. Thus, for a 10-mer, the probes correspond to bases 1 to 10, 2 to 11, 3 to 12 ... and so on. For a 15-mer, the probes correspond to bases 1 to 15, 2 to 16, 3 to 17 ... and so on. For a
25 20-mer, the probes correspond to bases 1 to 20, 2 to 21, 3 to 22 ... and so on.

It also will be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:1 and SEQ ID NO:2. Recombinant vectors and isolated DNA segments may therefore variously include the
30 IGFBP-2 coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that

nevertheless include IGFBP-2 -coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

The DNA segments of the present invention encompass biologically functional equivalent IGFBP-2 proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein.

If desired, one also may prepare fusion proteins and peptides, *e.g.*, where the IGFBP-2 coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (*e.g.*, proteins that may be purified by affinity chromatography and enzyme label coding regions, respectively).

20

Encompassed by certain embodiments of the present invention are DNA segments encoding relatively small peptides, such as, for example, peptides of from about 15 to about 50 amino acids in length, and more preferably, of from about 15 to about 30 amino acids in length; and also larger polypeptides up to and including proteins corresponding to the full-length sequences set forth in SEQ ID NO:2, or to specific fragments of SEQ ID NO:1 that correspond to differences as compared to the published sequence for IGFBP-2.

B. AMPLIFICATION AND PCR™

Nucleic acids used as a template for amplification are isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell

RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

5 Pairs of primers that selectively hybridize to nucleic acids corresponding to *IGFBP-2* are contacted with the isolated nucleic acid under conditions that permit selective hybridization. The term "primer", as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to
10 twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

15 Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

20 Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals.

25 A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCRTM) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and each incorporated herein by reference in entirety.

Briefly, in PCR™, two or more primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, e.g., *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

10

A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA 15 polymerases. These methods are described in WO 90/07641, filed December 21, 1990, incorporated herein by reference. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), 20 disclosed in EPA No. 320 308, incorporated herein by reference in its entirety. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, 25 incorporated herein by reference, also may be used as still another amplification method in the present invention.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 30 5'-[alpha-thio]-triphosphates in one strand of a restriction site also may be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair 5 Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences also can be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' 10 sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

15

Still another amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, 20 template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled 25 probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR Gingeras *et al.*, PCT Application WO 88/10315, incorporated 30 herein by reference.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

5

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This 10 scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, 1990 incorporated by reference).

Methods based on ligation of two (or more) oligonucleotides in the presence of 15 nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, also may be used in the amplification step of the present invention.

Following any amplification, it may be desirable to separate the amplification 20 product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See Sambrook *et al.*, 1989.

25 Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

30

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with

ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

5

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding 10 partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those 15 of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose or nylon, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of 20 hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U.S. Patent 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the 25 automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

4. RECOMBINANT VECTORS, HOST CELLS AND EXPRESSION

Recombinant vectors form important further aspects of the present invention. 30 The term "expression vector or construct" means any type of genetic construct

containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed and subsequently translated.

Particularly useful vectors are contemplated to be those vectors in which the
5 coding portion of the DNA segment, whether encoding a full length protein or smaller peptide, is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene.
10 The phrases "operatively positioned", "under control", "operably linked to" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The promoter may be in the form of the promoter that is naturally associated
15 with any of the *IGFBP-2* genes, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR technology, in connection with the compositions disclosed herein (PCR technology is disclosed in U.S. Patent 4,683,202 and U.S. Patent 4,683,195, each incorporated herein by reference).

20

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an *IGFBP-2* gene
25 in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell.

Naturally, it will be important to employ a promoter that effectively directs the
30 expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see

Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. In a preferred 5 embodiment, the promoter preferentially expresses or is specific for a tissue or cell type, such as a cell type that expresses IGFBP-2. It is contemplated that such cell types may be from, but not limited to, prostate and breast tissues, and may be altered or cancerous.

10 At least one module in a promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

15 Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements 20 frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

25 The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is 30 capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 3 and 4 below list several enhancers, promoters and inducible elements which may be employed, in the context of the present invention, to regulate the expression of any of the *IGFBP-2* constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of transgene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which serves as a binding region for one or more transcriptional proteins.

20

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

25 Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a transgene. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain

bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

Table 3
Promoter and Enhancer Elements

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl and Baltimore, 1985; Atchison and Perry, 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1984; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> ; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987; Winoto and Baltimore, 1989; Redondo <i>et al.</i> ; 1990
HLA DQ α and DQ β	Sullivan and Peterlin, 1987
β -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-DRA	Sherman <i>et al.</i> , 1989
β -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> ; 1989
Muscle Creatine Kinase	Jaynes <i>et al.</i> , 1988; Horlick and Benfield, 1989; Johnson <i>et al.</i> , 1989
Prealbumin (Transthyretin)	Costa <i>et al.</i> , 1988
Elastase I	Ornitz <i>et al.</i> , 1987
Metallothionein	Karin <i>et al.</i> , 1987; Culotta and Hamer, 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987

TABLE 3 - CONTINUED

Albumin Gene	Pinkert <i>et al.</i> , 1987; Tronche <i>et al.</i> , 1989, 1990
α -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β -Globin	Trudel and Constantini, 1987
e-fos	Cohen <i>et al.</i> , 1978
c-HA-ras	Triesman, 1985; Deschamps <i>et al.</i> , 1985
Insulin	Edlund <i>et al.</i> , 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh <i>et al.</i> , 1990
α_1 -Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy	Klamut <i>et al.</i> , 1990

TABLE 3 - CONTINUED

SV40	Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987; Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndall <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; de Villiers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a, b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander and Haseltine, 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Choi <i>et al.</i> , 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky and Botchan, 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987; Stephens and Hentschel, 1987; Gius <i>et al.</i> , 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988

TABLE 3 - CONTINUED

Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber and Cullan, 1988; Jakobovits <i>et al.</i> , 1988; Feng and Holland, 1988; Takebe <i>et al.</i> , 1988; Rosen <i>et al.</i> , 1988; Berkhout <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp and Marciniak, 1989; Braddock <i>et al.</i> , 1989
Cytomegalovirus	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

Table 4
Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA)	Palmiter <i>et al.</i> , 1982;
	Heavy metals	Haslinger and Karin, 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987, Karin <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987b; McNeall <i>et al.</i> , 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang <i>et al.</i> , 1981; Lee <i>et al.</i> , 1981; Majors and Varmus, 1983; Chandler <i>et al.</i> , 1983; Ponta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984

TABLE 4 - CONTINUED

Collagenase	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
SV40	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
α -2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2kb	Interferon	Blanar <i>et al.</i> , 1989
HSP70	Ela, SV40 Large T Antigen	Taylor <i>et al.</i> , 1989; Taylor and Kingston, 1990a, b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

Turning to the expression of IGFBP-2 polypeptides, once a suitable clone or clones have been obtained, whether they be cDNA based or genomic, one may proceed to prepare an expression system. The engineering of DNA segment(s) for expression in a prokaryotic or eukaryotic system may be performed by techniques generally known to those of skill in recombinant expression. It is believed that virtually any expression system may be employed in the expression of the proteins of the present invention.

Both cDNA and genomic sequences are suitable for eukaryotic expression, as the host cell will generally process the genomic transcripts to yield functional mRNA for translation into protein. Generally speaking, it may be more convenient to employ as the recombinant gene a cDNA version of the gene. It is believed that the use of a cDNA version will provide advantages in that the size of the gene will generally be

much smaller and more readily employed to transfect the targeted cell than will a genomic gene, which will typically be up to an order of magnitude larger than the cDNA gene. However, the inventors do not exclude the possibility of employing a genomic version of a particular gene where desired.

5

In expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation 10 signal and the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

15 A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that 20 the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. It is known that the primary sequence surrounding the ATG initiation codon $\text{GCC}(\text{^A}_\text{G})\text{CCATGG}$ is the optimal context for initiation of translation in higher eukaryotes (Kozak 1991). Thus 25 mutagenesis of the sequence surrounding the ATG codon may be mutated, as described in detail herein, is contemplated by the inventors as a mechanism to improve the efficiency of translation of the *IGFBP-2* constructs of the present invention.

As used herein, the terms "engineered" and "recombinant" cells are intended to 30 refer to a cell into which an exogenous DNA segment or gene, such as a cDNA or gene encoding an *IGFBP-2* polypeptide has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a

recombinantly introduced exogenous DNA segment or gene. Engineered cells are thus cells having a gene or genes introduced through the hand of man. Recombinant cells include those having an introduced cDNA or genomic gene, and also include genes positioned adjacent to a promoter not naturally associated with the particular 5 introduced gene.

To express a recombinant IGFBP-2 protein, whether mutant or wild-type, in accordance with the present invention one would prepare an expression vector that comprises a IGFBP-2-encoding nucleic acid under the control of one or more 10 promoters. To bring a coding sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded recombinant protein. This is the meaning of 15 "recombinant expression" in this context.

5. ANTISENSE AND RIBOZYMES

A. ANTISENSE

20 Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and 25 adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

30 Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target

polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

5

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs may include regions complementary to intron/exon splice junctions. Thus, antisense constructs with 10 complementarity to regions within 50-200 bases of an intron-exon splice junction may be used. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of 15 exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide 20 sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have 25 no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

30

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is

desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

5

B. RIBOZYMES

The use of IGFBP-2-specific ribozymes is claimed in the present application. The following information is provided in order to compliment the earlier section and to assist those of skill in the art in this endeavor.

10

Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlack *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with 15 a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

20

Ribozyme catalysis has primarily been observed as part of sequence specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U.S. Patent 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and 25 approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990; Sioud *et al.*, 1992). Recently, it was reported that ribozymes elicited genetic changes in some cell lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. 30 Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme. In light of the information included herein and the knowledge of one of ordinary skill in the art, the preparation

and use of additional ribozymes that are specifically targeted to a given gene will now be straightforward.

Several different ribozyme motifs have been described with RNA cleavage activity (reviewed in Symons, 1992). Examples that would be expected to function equivalently for the down regulation of IGFBP-2 include sequences from the Group I self splicing introns including tobacco ringspot virus (Prody *et al.*, 1986), avocado sunblotch viroid (Palukaitis *et al.*, 1979 and Symons, 1981), and Lucerne transient streak virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead ribozymes based on a predicted folded secondary structure.

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity (Yuan *et al.*, 1992, Yuan and Altman, 1994), hairpin ribozyme structures (Berzal-Herranz *et al.*, 1992; Chowrira *et al.*, 1993) and hepatitis δ virus based ribozymes (Perrotta and Been, 1992). The general design and optimization of ribozyme directed RNA cleavage activity has been discussed in detail (Haseloff and Gerlach, 1988, Symons, 1992, Chowrira, *et al.*, 1994, and Thompson, *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to a site by complimentary base pair interactions. Two stretches of homology are required for this targeting. These stretches of homologous sequences flank the catalytic ribozyme structure defined above. Each stretch of homologous sequence can vary in length from 7 to 15 nucleotides. The only requirement for defining the homologous sequences is that, on the target RNA, they are separated by a specific sequence which is the cleavage site. For hammerhead ribozymes, the cleavage site is a dinucleotide sequence on the target RNA, uracil (U) followed by either an adenine, cytosine or uracil (A,C or U; Perriman, *et al.*, 1992; Thompson, *et al.*, 1995). The frequency of this dinucleotide occurring in any given RNA is statistically 3 out of 16. Therefore, for a given target messenger RNA of 1000 bases, 187 dinucleotide cleavage sites are statistically possible. The message for IGFBP-2 targeted here are greater than 1400 bases long, with greater than 260 possible cleavage sites.

The large number of possible cleavage sites in the IGFBP-2 coupled with the growing number of sequences with demonstrated catalytic RNA cleavage activity indicates that a large number of ribozymes that have the potential to downregulate the 5 IGFBP-2 are available. Designing and testing ribozymes for efficient cleavage of a target RNA is a process well known to those skilled in the art. Examples of scientific methods for designing and testing ribozymes are described by Chowrira *et al.*, (1994) and Lieber and Strauss (1995), each incorporated by reference. The identification of operative and preferred sequences for use in IGFBP-2-targeted ribozymes is simply a 10 matter of preparing and testing a given sequence, and is a routinely practiced "screening" method known to those of skill in the art.

C. SINGLE-CHAIN ANTIBODIES

Another therapeutic embodiment of the present invention contemplates the use 15 of single-chain antibodies to block the activity of IGFBP-2 in cells, in particular cancer cells. Single-chain antibodies can be synthesized by a cell, targeted to particular cellular compartments, and used to interfere in a highly specific manner with cell growth and metabolism (Richardson and Marasco, 1995). Recently, single-chain antibodies were utilized for the phenotypic knockout of growth-factor receptors, the 20 functional inactivation of p21ras, and the inhibition of HIV-1 replication. Intracellular antibodies offer a simple and effective alternative to other forms of gene inactivation, as well as demonstrate a clear potential as reagents for cancer therapy and for the control of infectious diseases. Single-chain antigen-binding proteins also represent potentially unique molecules for targeted delivery of drugs, toxins, or radionuclides to 25 a tumor site, and show increased accessibility to tumor cells *in vivo* (Yokoda *et al.*, 1992). Single-chain antibodies that bind IGFBP-2 can be introduced into a cell to functionally inactivate the IGFBP-2.

Methods for the production of single-chain antibodies are well known to those 30 of skill in the art. The skilled artisan is referred to U.S. Patent 5,359,046, (incorporated herein by reference) for such methods. A single-chain antibody is

created by fusing together the variable domains of the heavy and light chains using a short peptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (scFvs) in which the C-terminus of one variable domain is tethered to the N-terminus of the other *via* a 15 to 25 amino acid peptide or linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk *et al.*, 1990; Chaudhary *et al.*, 1990). These Fvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody.

10

It is also contemplated by the present invention that single-chain antibody therapy can be combined with chemotherapeutic or radiotherapeutic intervention. The discussion of combined therapy with traditional chemotherapy or radiotherapy employed herein is incorporated into this section by reference.

15

6. METHODS OF GENE TRANSFER

In order to mediate the effect transgene expression in a cell, it will be necessary to transfer the therapeutic expression constructs of the present invention into a cell. Such transfer may employ viral or non-viral methods of gene transfer. This section 20 provides a discussion of methods and compositions of gene transfer.

A. Viral Vector-Mediated Transfer

The IGFBP-2 genes are incorporated into an adenoviral infectious particle to mediate gene transfer to a cell. Additional expression constructs encoding other 25 therapeutic agents as described herein may also be transferred *via* viral transduction using infectious viral particles, for example, by transformation with an adenovirus vector of the present invention as described herein below. Alternatively, retroviral or bovine papilloma virus may be employed, both of which permit permanent transformation of a host cell with a gene(s) of interest. Thus, in one example, viral 30 infection of cells is used in order to deliver therapeutically significant genes to a cell. Typically, the virus simply will be exposed to the appropriate host cell under physiologic conditions, permitting uptake of the virus. Though adenovirus is

exemplified, the present methods may be advantageously employed with other viral vectors, as discussed below.

i) Adenovirus.

5 Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized DNA genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. The roughly 36 kB viral genome is bounded by 100-200 base pair (bp) inverted terminal repeats (ITR), in which are contained *cis*-acting elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions
10 of the genome that contain different transcription units are divided by the onset of viral DNA replication.

The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2
15 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression, and host cell shut off (Renan, 1990). The products of the late genes (L1, L2, L3, L4 and L5), including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter
20 (MLP). The MLP (located at 16.8 map units) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5' tripartite leader (TL) sequence which makes them preferred mRNAs for translation.

In order for adenovirus to be optimized for gene therapy, it is necessary to
25 maximize the carrying capacity so that large segments of DNA can be included. It also is very desirable to reduce the toxicity and immunologic reaction associated with certain adenoviral products. The two goals are, to an extent, coterminous in that elimination of adenoviral genes serves both ends. By practice of the present invention, it is possible achieve both these goals while retaining the ability to manipulate the
30 therapeutic constructs with relative ease.

The large displacement of DNA is possible because the *cis* elements required for viral DNA replication all are localized in the inverted terminal repeats (ITR) (100-200 bp) at either end of the linear viral genome. Plasmids containing ITR's can replicate in the presence of a non-defective adenovirus (Hay *et al.*, 1984). Therefore, 5 inclusion of these elements in an adenoviral vector should permit replication.

In addition, the packaging signal for viral encapsidation is localized between 194-385 bp (0.5-1.1 map units) at the left end of the viral genome (Hearing *et al.*, 1987). This signal mimics the protein recognition site in bacteriophage λ DNA where 10 a specific sequence close to the left end, but outside the cohesive end sequence, mediates the binding to proteins that are required for insertion of the DNA into the head structure. E1 substitution vectors of Ad have demonstrated that a 450 bp (0-1.25 map units) fragment at the left end of the viral genome could direct packaging in 293 cells (Levrero *et al.*, 1991).

15

Previously, it has been shown that certain regions of the adenoviral genome can be incorporated into the genome of mammalian cells and the genes encoded thereby expressed. These cell lines are capable of supporting the replication of an adenoviral 20 vector that is deficient in the adenoviral function encoded by the cell line. There also have been reports of complementation of replication deficient adenoviral vectors by "helping" vectors, *e.g.*, wild-type virus or conditionally defective mutants.

Replication-deficient adenoviral vectors can be complemented, in *trans*, by 25 helper virus. This observation alone does not permit isolation of the replication-deficient vectors, however, since the presence of helper virus, needed to provide replicative functions, would contaminate any preparation. Thus, an additional element was needed that would add specificity to the replication and/or packaging of the replication-deficient vector. That element, as provided for in the present invention, derives from the packaging function of adenovirus.

30

It has been shown that a packaging signal for adenovirus exists in the left end of the conventional adenovirus map (Tibbetts, 1977). Later studies showed that a

mutant with a deletion in the E1A (194-358 bp) region of the genome grew poorly even in a cell line that complemented the early (E1A) function (Hearing and Shenk, 1983). When a compensating adenoviral DNA (0-353 bp) was recombined into the right end of the mutant, the virus was packaged normally. Further mutational analysis 5 identified a short, repeated, position-dependent element in the left end of the Ad5 genome. One copy of the repeat was found to be sufficient for efficient packaging if present at either end of the genome, but not when moved towards the interior of the Ad5 DNA molecule (Hearing *et al.*, 1987).

10 By using mutated versions of the packaging signal, it is possible to create helper viruses that are packaged with varying efficiencies. Typically, the mutations are point mutations or deletions. When helper viruses with low efficiency packaging are grown in helper cells, the virus is packaged, albeit at reduced rates compared to wild-type virus, thereby permitting propagation of the helper. When these helper viruses are 15 grown in cells along with virus that contains wild-type packaging signals, however, the wild-type packaging signals are recognized preferentially over the mutated versions. Given a limiting amount of packaging factor, the virus containing the wild-type signals are packaged selectively when compared to the helpers. If the preference is great enough, stocks approaching homogeneity should be achieved.

20

ii) Retrovirus.

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into 25 cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes - gag, pol and env - that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene, termed Ψ , functions as a signal for 30 packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and

enhancer sequences and also are required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a promoter is
5 inserted into the viral genome in the place of certain viral sequences to produce a virus
that is replication-defective. In order to produce virions, a packaging cell line
containing the gag, pol and env genes but without the LTR and Ψ components is
constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a human
cDNA, together with the retroviral LTR and Ψ sequences is introduced into this cell
10 line (by calcium phosphate precipitation for example), the Ψ sequence allows the RNA
transcript of the recombinant plasmid to be packaged into viral particles, which are
then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986;
Mann *et al.*, 1983). The media containing the recombinant retroviruses is collected,
optionally concentrated, and used for gene transfer. Retroviral vectors are able to
15 infect a broad variety of cell types. However, integration and stable expression of
many types of retroviruses require the division of host cells (Paskind *et al.*, 1975).

An approach designed to allow specific targeting of retrovirus vectors recently
was developed based on the chemical modification of a retrovirus by the chemical
20 addition of galactose residues to the viral envelope. This modification could permit the
specific infection of cells such as hepatocytes *via* asialoglycoprotein receptors, should
this be desired.

A different approach to targeting of recombinant retroviruses was designed in
25 which biotinylated antibodies against a retroviral envelope protein and against a
specific cell receptor were used. The antibodies were coupled *via* the biotin
components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major
histocompatibility complex class I and class II antigens, the infection of a variety of
human cells that bore those surface antigens was demonstrated with an ecotropic virus
30 *in vitro* (Roux *et al.*, 1989).

iii) Adeno-associated Virus.

AAV utilizes a linear, single-stranded DNA of about 4700 base pairs. Inverted terminal repeats flank the genome. Two genes are present within the genome, giving rise to a number of distinct gene products. The first, the *cap* gene, produces three 5 different virion proteins (VP), designated VP-1, VP-2 and VP-3. The second, the *rep* gene, encodes four non-structural proteins (NS). One or more of these *rep* gene products is responsible for transactivating AAV transcription.

10 The three promoters in AAV are designated by their location, in map units, in the genome. These are, from left to right, p5, p19 and p40. Transcription gives rise to six transcripts, two initiated at each of three promoters, with one of each pair being spliced. The splice site, derived from map units 42-46, is the same for each transcript. The four non-structural proteins apparently are derived from the longer of the transcripts, and three virion proteins all arise from the smallest transcript.

15

AAV is not associated with any pathologic state in humans. Interestingly, for efficient replication, AAV requires "helping" functions from viruses such as herpes simplex virus I and II, cytomegalovirus, pseudorabies virus and, of course, adenovirus. The best characterized of the helpers is adenovirus, and many "early" functions for this 20 virus have been shown to assist with AAV replication. Low level expression of AAV *rep* proteins is believed to hold AAV structural expression in check, and helper virus infection is thought to remove this block.

25 The terminal repeats of the AAV vector can be obtained by restriction endonuclease digestion of AAV or a plasmid such as p201, which contains a modified AAV genome (Samulski *et al.* 1987), or by other methods known to the skilled artisan, including but not limited to chemical or enzymatic synthesis of the terminal repeats based upon the published sequence of AAV. The ordinarily skilled artisan can determine, by well-known methods such as deletion analysis, the minimum sequence or 30 part of the AAV ITRs which is required to allow function, *i.e.*, stable and site-specific integration. The ordinarily skilled artisan also can determine which minor

modifications of the sequence can be tolerated while maintaining the ability of the terminal repeats to direct stable, site-specific integration.

AAV-based vectors have proven to be safe and effective vehicles for gene delivery *in vitro*, and these vectors are being developed and tested in pre-clinical and clinical stages for a wide range of applications in potential gene therapy, both *ex vivo* and *in vivo* (Flotte and Carter, 1995 ; Chatterjee *et al.*, 1995; Ferrari *et al.*, 1996; Fisher *et al.*, 1996; Flotte *et al.*, 1993; Goodman *et al.*, 1994; Kaplitt *et al.*, 1994; 1996, Kessler *et al.*, 1996; Koeberl *et al.*, 1997; Mizukami *et al.*, 1996; Xiao *et al.*, 10 1996).

AAV-mediated efficient gene transfer and expression in the lung has led to clinical trials for the treatment of cystic fibrosis (Flotte and Carter, 1995; Flotte *et al.*, 1993). Similarly, the prospects for treatment of muscular dystrophy by AAV-mediated gene delivery of the dystrophin gene to skeletal muscle, of Parkinson's disease by tyrosine hydroxylase gene delivery to the brain, of hemophilia B by Factor IX gene delivery to the liver, and potentially of myocardial infarction by vascular endothelial growth factor gene to the heart, appear promising since AAV-mediated transgene expression in these organs has recently been shown to be highly efficient (Fisher *et al.*, 15 1996; Flotte *et al.*, 1993; Kaplitt *et al.*, 1994; 1996; Koeberl *et al.*, 1997; McCown *et al.*, 1996; Ping *et al.*, 1996; Xiao *et al.*, 1996).

iv) Other Viral Vectors.

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) canary pox virus, Ebstein Barr Virus, and herpesviruses may be employed. These viruses offer several features for use in gene transfer into various mammalian cells.

B. Non-viral Transfer

DNA constructs of the present invention are generally delivered to a cell, in certain situations, the nucleic acid to be transferred is non-infectious, and can be transferred using non-viral methods.

5

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979), cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988).

15

Once the construct has been delivered into the cell the nucleic acid encoding the therapeutic gene may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the therapeutic gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In a particular embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution.

The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). The addition of DNA to cationic liposomes causes a topological transition from liposomes to optically birefringent liquid-crystalline 5 condensed globules (Radler *et al.*, 1997). These DNA-lipid complexes are potential non-viral vectors for use in gene therapy.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Using the β -lactamase gene, Wong *et al.* (1980) 10 demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa, and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection. Also included are various commercial approaches involving "lipofection" technology.

15

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in 20 conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention.

25

Other vector delivery systems which can be employed to deliver a nucleic acid encoding a therapeutic gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution 30 of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and 5 transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

10 In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a therapeutic gene also may be specifically delivered into a cell 15 type such as prostate, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, the human prostate-specific antigen (Watt *et al.*, 1986) may be used as the receptor for mediated delivery of a nucleic acid in prostate tissue.

20 In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is applicable particularly for transfer *in vitro*, however, it may be applied for *in vivo* use as well. Dubensky *et al.* (1984) successfully 25 injected polyomavirus DNA in the form of CaPO₄ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of CaPO₄ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a CAM may also be transferred in a similar manner *in* 30 *vivo* and express CAM.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads

10 **7. IMMUNOTHERAPY**

The results presented herein have significant relevance to immunotherapy of human diseases and disorders, including cancer. In using the immunotherapeutic compositions derived from the IGFBP-2 polypeptides and peptides of the present invention in treatment methods, other standard treatments also may be employed, such 15 as radiotherapy or chemotherapy. However, it is preferred that the immunotherapy be used alone initially as its effectiveness can be readily assessed. Immunotherapies of cancer can broadly be classified as adoptive, passive and active, as described in the following sections.

20 It is contemplated that a wide variety of cancers may be treated using therapies described herein. Such cancers include but are not limited to cancers of the brain (*e.g.*, glioblastoma, astrocytoma, oligodendrogloma, ependymomas), neurofibrosarcoma, meningia, lung, liver, spleen, kidney, lymph node, pancreas, small intestine, blood cells, colon or colorectal region, stomach, thyroid, breast, endometrium, prostate, testes, 25 ovary, skin, head and neck, esophagus, bone marrow, blood or other tissue.

A. PASSIVE IMMUNOTHERAPY

A number of different approaches for passive immunotherapy of cancer exist. They may be broadly categorized into the following: injection of antibodies alone; 30 injection of antibodies coupled to toxins or chemotherapeutic agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotype antibodies; and finally, purging of tumor cells in bone marrow.

Preferably, human monoclonal antibodies are employed in passive immunotherapy, as they produce few or no side effects in the patient. However, their application is somewhat limited by their scarcity and have so far only been
5 administered intralesionally. Human monoclonal antibodies to ganglioside antigens have been administered intralesionally to patients suffering from cutaneous recurrent melanoma (Irie & Morton, 1986). Regression was observed in six out of ten patients, following, daily or weekly, intralesional injections. In another study, moderate success was achieved from intralesional injections of two human monoclonal antibodies (Irie *et al.*, 1989).

It may be favorable to administer more than one monoclonal antibody directed against two different antigens or even antibodies with multiple antigen specificity. Treatment protocols also may include administration of lymphokines or other immune
15 enhancers as described by Bajorin *et al.* (1988). The development of human monoclonal antibodies is described in further detail elsewhere in the specification.

B. ACTIVE IMMUNOTHERAPY

In active immunotherapy, an antigenic peptide, polypeptide or protein, or an
20 autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath & Morton, 1991; Morton & Ravindranath, 1996; Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993). In melanoma immunotherapy, those patients who elicit high IgM response often survive better than those who elicit no or low IgM antibodies (Morton *et al.*, 1992).
25 IgM antibodies are often transient antibodies and the exception to the rule appears to be anti-ganglioside or ant(carbohydrate) antibodies.

C. ADOPTIVE IMMUNOTHERAPY

In adoptive immunotherapy, the patient's circulating lymphocytes, or tumor
30 infiltrated lymphocytes, are isolated *in vitro*, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered (Rosenberg *et al.*, 1988; 1989). To achieve this, one would administer to an animal, or human patient, an

immunologically effective amount of activated lymphocytes in combination with an adjuvant-incorporated antigenic peptide composition as described herein. The activated lymphocytes will most preferably be the patient's own cells that were earlier isolated from a blood or tumor sample and activated (or "expanded") *in vitro*. This form of immunotherapy has produced several cases of regression of melanoma and renal carcinoma, but the percentage of responders were few compared to those who did not respond.

8. METHODS OF TREATING CANCER

In a particular aspect, the present invention provides methods for the treatment of various malignancies involving IGFBP-2 directed therapy, which includes compounds or compositions that affect IGFBP-2. Treatment methods will involve treating an individual with an effective amount of a viral particle, as described above, containing a therapeutic gene of interest. Other treatment methods involve treating an individual with an effective amount of an antibody. Alternatively, treatment may involve administering an IGFBP-2 peptide or polypeptide that elicits an immune response, which effects similar results as administration of an anti-IGFBP-2 antibody. An effective amount is described, generally, as that amount sufficient to detectably and repeatedly ameliorate, reduce, minimize or limit the extent of a disease or its symptoms. More rigorous definitions may apply, including elimination, eradication or cure of disease. Target cancer cells include cancers of the lung, brain, prostate, kidney, liver, ovary, breast, skin, stomach, esophagus, head and neck, testicles, colon, cervix, lymphatic system and blood. Of particular interest are cancers of the prostate.

To kill cells, inhibit cell growth, inhibit metastasis, decrease tumor size and otherwise reverse or reduce the malignant phenotype of tumor cells, using the methods and compositions of the present invention, one would generally contact a patient with the IGFBP-2 directed therapy. This may be combined with surgery or compositions comprising other agents effective in the treatment of cancer. Thus it is contemplated that the IGFBP-2 directed therapy may be combined with other anti-cancer therapies or agents, such as surgery, immunotherapy, chemotherapy, radiotherapy, gene therapy, or hormone therapy. These compositions would be provided in a combined amount

effective to kill or inhibit proliferation of a cancer cell. This process may involve contacting the cells with the IGFBP-2 directed therapy and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell 5 with two distinct compositions or formulations, at the same time, wherein one composition includes the IGFBP-2 directed therapy and the other includes the second agent.

Alternatively, the IGFBP-2 directed therapy may precede or follow the other 10 agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and IGFBP-2 directed therapy are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and IGFBP-2 directed therapy would still be able to exert an advantageously combined effect on the cell. In such instances, it is 15 contemplated that one would contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other, with a delay time of only about 12 h being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective 20 administrations.

i) Chemotherapy

Cancer therapies can include a variety of combination therapies with both 25 chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin and methotrexate or any analog or derivative variant thereof.

30

ii) Radiation Therapy

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

iii) Surgery

Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and miscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

iv) Gene Therapy

In yet another embodiment, the secondary treatment is gene therapy in which a therapeutic polynucleotide is administered before, after, or at the same time as the IGFBP-2 directed agent. Genes encoding proteins that induce cellular proliferation, that inhibit cell proliferation, or that regulate apoptosis are especially useful in the context of the present invention. Genes encoding proteins that induce cellular

proliferation include erbA, erbB, fos, jun, myc, src, ras, abl, fms, neu, her, myb, and rel. In one embodiment of the present invention, it is contemplated that anti-sense mRNA directed to a particular inducer of cellular proliferation is used to prevent expression of the inducer of cellular proliferation. Genes that encode inhibitors of cell 5 proliferation include genes known as tumor suppressor genes. Examples are p53, p16, Rb, CCAM, APC, DCC, NF-1, NF-2, and WT-1. Many proteins involved in apoptosis are in the Bcl family. Some of these act like Bcl-2 (e.g., Bcl_{XL}, Bcl_w, Bcl_s, Mcl-1, A1, Bfl-1), while others counteract Bcl-2 function and promote cell death (e.g., Bax, Bak, Bik, Bim, Bid, Bad, Harakiri). The genes encoding proteins involved in apoptosis are 10 contemplated for use with compositions of the present invention. Other genes that may be employed in an anti-cancer therapy with an IGFBP-2 directed agent include MEN-I, MEN-II, zac1, p73, VHL, MMAC1 / PTEN, DBCCR-1, FCC, rsk-3, p27, p27/p16 fusions, p21/p27 fusions, anti-thrombotic genes (e.g., COX-1, TFPI), PGS, Dp, E2F, *trk*, *ret*, *gsp*, *hst*, E1A, p300, genes involved in angiogenesis (e.g., VEGF, 15 FGF, thrombospondin, BAI-1, GDAIF, or their receptors) and MCC.

Finally, it is contemplated that the other IGFBP family members such as IGFBP-1, -3, -4, or -5, may be used in gene therapy methods in combination with an 20 IGFBP-2 directed therapy. For example, the level of IGFBP-3 may be modulated in conjunction with the administration of an IGFBP-2 directed therapy. Also, the level of proteins implicated in the regulation of IGFBPs, such as prostate-specific antigen (PSA) may also be altered in a combination treatment. Levels of PSA may be reduced by any of the methods described herein, such as by antisense of antibody modulation, or a vaccine against PSA may be used. See U.S. Patent No. 5,925,362, which is 25 hereby incorporated by reference. Alternatively, genes involved in IGFBP modes of action, for example, IGF-II-R, IGFs, IGF-I-R may also be similarly employed.

v) *Hormonal Therapy*

Hormonal therapy may also be used in conjunction with the present invention 30 or in combination with any other cancer therapy previously described. The use of hormones may be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones

such as testosterone or estrogen (*e.g.*, anti-androgen or anti-estrogen agents). This treatment is often used in combination with at least one other cancer therapy as a treatment option or to reduce the risk of metastases.

5

vi) Other Anti-Cancer Therapies

Another form of therapy for use in conjunction with chemotherapy, radiation therapy or biological therapy includes hyperthermia, which is a procedure in which a patient's tissue is exposed to high temperatures (up to 106°F). External or internal heating devices may be involved in the application of local, regional, or whole-body 10 hyperthermia. Local hyperthermia involves the application of heat to a small area, such as a tumor. Heat may be generated externally with high-frequency waves targeting a tumor from a device outside the body. Internal heat may involve a sterile probe , including thin, heated wires or hollow tubes filled with warm water, implanted microwave antennae, or radiofrequency electrodes.

15

A patient's organ or a limb is heated for regional therapy, which is accomplished using devices that produce high energy, such as magnets. Alternatively, some of the patient's blood may be removed and heated before being perfused into an area that will be internally heated. Whole-body heating may also be implemented in 20 cases where cancer has spread throughout the body. Warm-water blankets, hot wax, inductive coils, and thermal chambers may be used for this purpose.

Various combinations may be employed, for instance, where the IGFB-2 directed agent is "A" and the second anti-cancer therapy is "B":

25

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B

B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

30

B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell
5 in a combined amount effective to kill the cell or prevent it from dividing.

Administration of the IGFBP-2 directed therapy of the present invention to a patient will follow general protocols for the administration of chemotherapeutics, taking into account the toxicity, if any, of the therapies. It is expected that the
10 treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described IGFBP-2 directed therapies.

Depending on the particular cancer to be treated, administration of therapeutic
15 compositions according to the present invention will be *via* any common route so long as the target tissue is available *via* that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Topical administration would be particularly advantageous for treatment of skin cancers. Alternatively, administration will be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection.
20 Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients.

In certain embodiments, *ex vivo* therapies also are contemplated. *Ex vivo*
25 therapies involve the removal, from a patient, of target cells. The cells are treated outside the patient's body and then returned. One example of *ex vivo* therapy would involve a variation of autologous bone marrow transplant. Many times, ABMT fails because some cancer cells are present in the withdrawn bone marrow, and return of the bone marrow to the treated patient results in repopulation of the patient with cancer
30 cells. In one embodiment, however, the withdrawn bone marrow cells could be treated while outside the patient with a viral particle that targets and kills the cancer cell. Once the bone marrow cells are "purged," they can be reintroduced into the patient.

The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. Also of importance is the subject to be treated, in particular, the state of the subject and the protection desired. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of plaque forming units (pfu) of the viral construct. Unit doses range from 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} pfu and higher.

One of the preferred embodiments of the present invention involves the use of viral vectors to deliver therapeutic genes to cancer cells. According to the present invention, one may treat the cancer by directly injection a tumor with the viral vector. Alternatively, the tumor may be infused or perfused with the vector using any suitable delivery vehicle. Local or regional administration, with respect to the tumor, also is contemplated. Finally, systemic administration may be performed. Continuous administration also may be applied where appropriate, for example, where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease. Delivery *via* syringe or catheterization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition *via* continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

For tumors of > 4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of < 4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to

about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals.

Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may 5. be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

10

In certain embodiments, the tumor being treated may not, at least initially, be resectable. Treatments with therapeutic viral constructs may increase the resectability 15 of the tumor due to shrinkage at the margins or by elimination of certain particularly invasive portions. Following treatments, resection may be possible. Additional viral treatments subsequent to resection will serve to eliminate microscopic residual disease at the tumor site.

A typical course of treatment, for a primary tumor or a post-excision tumor bed, will involve multiple doses. Typical primary tumor treatment involves a 6 dose 20 application over a two-week period. The two-week regimen may be repeated one, two, three, four, five, six or more times. During a course of treatment, the need to complete the planned dosing may be re-evaluated.

9. PHARMACEUTICAL COMPOSITIONS

25 Aqueous compositions of the present invention comprise an effective amount of the IGFBP-2 directed therapeutic dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an 30 animal, or a human, as appropriate.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. For human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

10

The biological material should be extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle, where appropriate. The active compounds will then generally be formulated for parenteral administration, *e.g.*, formulated for injection via the intravenous, intramuscular, sub-cutaneous, intralesional, or even intraperitoneal routes. The preparation of an aqueous composition that contains an RBP agent as an active component or ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

30

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as

hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

5

An IGFBP-2 directed agent of the present invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. In terms of using peptide therapeutics as active ingredients, the technology of U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, each incorporated herein by reference, may be used.

The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

30

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally,

dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are
5 vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparation of more, or highly, concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active
10 agents to a small area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of
15 injectable solutions described above, but drug release capsules and the like also can be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic
20 with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to
25 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

30

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms

include, e.g., tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used, including creams.

One may also use nasal solutions or sprays, aerosols or inhalants in the present
5 invention. Nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays. Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, the aqueous nasal solutions usually are isotonic and slightly buffered to maintain a pH of 5.5 to 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic
10 preparations, and appropriate drug stabilizers, if required, may be included in the formulation. Various commercial nasal preparations are known and include, for example, antibiotics and antihistamines and are used for asthma prophylaxis.

Additional formulations which are suitable for other modes of administration
15 include suppositories and pessaries. A rectal pessary or suppository may also be used. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum, vagina or the urethra. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be
20 formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%.

Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium
25 saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. In certain defined embodiments, oral pharmaceutical compositions will comprise an inert diluent or assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be
30 incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers,

and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 75% of the weight of the unit, or preferably between 25-60%. The amount of active compounds 5 in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following:
a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as
10 dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier.
15 Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor.

20

10. Kits

Modulators of IGFBP-2 dependent cells can be assembled into kits. These kits can contain antibody compositions that recognize IGFBP-2, recombinant vectors containing a promoter operably linked to a nucleic acid segment that encodes IGFBP-
25 2, or any other modulators as described or anticipated in this application. All the essential materials and reagents required for inhibiting an IGFBP-2 dependent tumor cell may be assembled together in a kit. When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being particularly preferred.

30

For *in vivo* use, a chemotherapeutic agent may be formulated into a single or separate pharmaceutically acceptable syringeable composition. In this case, the

container means may itself be an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the lungs, injected into an animal, or even applied to and mixed with the other components of the kit.

5

The components of the kit may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means. The kits of the invention may also include an 10 instruction sheet defining administration of the gene therapy and/or the chemotherapeutic drug.

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, e.g., injection or 15 blow-molded plastic containers into which the desired vials are retained. Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalant, syringe, pipette, forceps, measured spoon, eye dropper 20 or any such medically approved delivery vehicle.

11. Methods for Screening Active Compounds

The present invention also contemplates the use of IGFBP-2 dependent cells, 25 IGFBP-2 and active fragments, and nucleic acids coding thereof, in the screening of compounds for activity in inhibiting an IGFBP-2 dependent cell. These assays may make use of a variety of different formats and may depend on the kind of "activity" for which the screen is being conducted. Contemplated functional "read-outs" include binding to a compound, inhibition of binding to a substrate, ligand, receptor or other binding partner by a compound, phosphatase activity, anti-phosphatase activity, 30 phosphorylation of IGFBP-2, dephosphorylation of IGFBP-2, inhibition or stimulation of cell-to-cell signaling, growth, metastasis, cell division, cell migration, soft agar

colony formation, contact inhibition, invasiveness, angiogenesis, apoptosis, tumor progression or other malignant phenotype.

A. IN VITRO ASSAYS

5 In one embodiment, the invention is to be applied for the screening of compounds that bind to the IGFBP-2 peptide or polypeptide. The polypeptide or fragment may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the polypeptide or the compound may be labeled, thereby permitting determining of binding.

10

In another embodiment, the assay may measure the inhibition of binding of IGFBP-2 to a natural or artificial substrate or binding partner. Competitive binding assays can be performed in which one of the agents (IGFBP-2, binding partner or compound) is labeled. Usually, the polypeptide will be the labeled species. One may 15 measure the amount of free label versus bound label to determine binding or inhibition of binding.

20 In another embodiment, the assay may measure the inhibition of IGFBP-2 transcription, translation or secretion. These assays will generally be cell-based assays where detection of the secreted IGFBP-2 is monitored. A decrease in IGFBP-2 in the presence of a candidate substance compared to the levels of IGFBP-2 in the absence of a candidate substance would be screened for.

25 Another technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with IGFBP-2 and washed. Bound polypeptide is detected by various methods.

30 Purified IGFBP-2 can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to the polypeptide can be used to immobilize the polypeptide to a solid phase. Also,

fusion proteins containing a reactive region (preferably a terminal region) may be used to link the IGFBP-2 active region to a solid phase.

Various cell lines containing wild-type or natural or engineered mutations in
5 IGFBP-2 can be used to study various functional attributes of IGFBP-2 and how a candidate compound affects these attributes. Methods for engineering mutations are described elsewhere in this document, as are naturally-occurring alleles of IGFBP-2. In such assays, the compound would be formulated appropriately, given its biochemical nature, and contacted with a target cell. Depending on the assay, culture
10 may be required. The cell may then be examined by virtue of a number of different physiologic assays. Alternatively, molecular analysis may be performed in which the function of IGFBP-2, or related pathways, may be explored. This may involve assays such as those for protein expression, enzyme function, substrate utilization, phosphorylation states of various molecules including IGFBP-2, cAMP levels, mRNA
15 expression (including differential display of whole cell or polyA RNA) and others.

B. IN VIVO ASSAYS

The present invention also encompasses the use of various animal models. Here, the identity seen between human and rat IGFBP-2 provides an excellent
20 opportunity to examine the function of IGFBP-2 in a whole animal system where it is normally expressed. By developing or isolating mutant cells lines that fail to express or overexpress normal IGFBP-2, one can generate cancer models in rats that will be highly predictive of cancers in humans and other mammals. These models may employ the orthotopic or systemic administration of tumor cells to mimic primary and/or
25 metastatic cancers. Alternatively, one may induce cancers in animals by providing agents known to be responsible for certain events associated with malignant transformation and/or tumor progression. Finally, transgenic animals (discussed below) that lack a wild-type IGFBP-2 may be utilized as models for cancer development and treatment.

30

Treatment of animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any

route that could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated are
5 systemic intravenous injection, regional administration via blood or lymph supply and intratumoral injection.

Determining the effectiveness of a compound *in vivo* may involve a variety of different criteria. Such criteria include, but are not limited to, survival, reduction of
10 tumor burden or mass, arrest or slowing of tumor progression, elimination of tumors, inhibition or prevention of metastasis, increased activity level, improvement in immune effector function and improved food intake.

15 **C. RATIONAL DRUG DESIGN**

The goal of rational drug design is to produce structural analogs of biologically active polypeptides or compounds with which they interact (agonists, antagonists, inhibitors, binding partners, *etc.*). By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different
20 susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for IGFBP-2 or a fragment thereof. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches. An alternative approach, "alanine scan," involves the random replacement of residues throughout molecule with alanine,
25 and the resulting effect on function determined.

It also is possible to isolate a IGFBP-2 specific antibody, selected by a functional assay, and then solve its crystal structure. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to
30 bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotype would be expected to be an analog of the original

antigen. The anti-idiotype could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

5

Thus, one may design drugs which have improved IGFBP-2 activity or which act as stimulators, inhibitors, agonists, antagonists or IGFBP-2 or molecules affected by IGFBP-2 function. By virtue of the availability of cloned IGFBP-2 sequences, sufficient amounts of IGFBP-2 can be produced to perform crystallographic studies.

10 In addition, knowledge of the polypeptide sequences permits computer employed predictions of structure-function relationships.

12. BIOLOGICAL FUNCTIONAL EQUIVALENTS

As modifications and changes may be made in the structure of IGFBP-2 genes
15 and proteins of the present invention, and still obtain molecules having like or otherwise desirable characteristics, such biologically functional equivalents are also encompassed within the present invention.

For example, certain amino acids may be substituted for other amino acids in a
20 protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies, binding sites on substrate molecules or receptors, DNA binding sites, or such like. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein
25 sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein with like (agonistic) properties. It is thus contemplated by the inventors that various changes may be made in the sequence of IGFBP-2 proteins or polypeptides, or underlying DNA, without appreciable loss of their biological utility or activity.

30 In terms of functional equivalents, it is well understood by the skilled artisan that, inherent in the definition of a "biologically functional equivalent protein or peptide or gene", is the concept that there is a limit to the number of changes that may be made

within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted.

5

In particular, where shorter length peptides are concerned, it is contemplated that fewer amino acid substitutions should be made within the given peptide. Longer domains may have an intermediate number of changes. The full length protein will have the most tolerance for a larger number of changes. Of course, a plurality of 10 distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention.

It also is well understood that where certain residues are shown to be particularly important to the biological or structural properties of a protein or peptide, 15 e.g., residues in binding regions or active sites, such residues may not generally be exchanged. This is an important consideration in the present invention, where changes in the IGFBP-2 should be carefully considered and subsequently tested to ensure maintenance of biological function, where maintenance of biological function is desired. In this manner, functional equivalents are defined herein as those peptides 20 which maintain a substantial amount of their native biological activity.

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid 25 side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically 30 functional equivalents.

To effect more quantitative changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); 5 alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

10 The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ± 2 is 15 preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

20 It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biological functional equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in certain embodiments of the present invention. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.* with a biological 25 property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have 30 been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 \pm 1); glutamate (+3.0 \pm 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 \pm 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

While discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes may be effected by alteration of the encoding DNA; taking into consideration also that the genetic code is degenerate and that two or more codons may code for the same amino acid. A table of amino acids and their codons is presented herein for use in such embodiments, as well as for other uses, such as in the design of probes and primers and the like.

In addition to the IGFBP-2 peptidyl compounds described herein, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure or to interact specifically with, for example, substrates or receptor compounds. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and hence are also functional equivalents.

20

Certain mimetics that mimic elements of protein secondary structure are described in Johnson *et al.* (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orientate amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is thus designed to permit molecular interactions similar to the natural molecule.

13. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to

constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5

EXAMPLE 1

The Effect of IGF, IGFBP-2 and Antibodies to IGFBP-2 on Growth and Gene Expression in Prostate Cell Lines

10 A. MATERIALS AND METHODS

Reagents: Rat IGFBP-2 is purchased from Research Diagnostics, Inc. (Flanders, NJ), and rat IGFBP-2 antibody is custom-made for the experiments by Genemed Synthesis Inc. (San Francisco, CA). A polyclonal antibody against human IGFBP-2 was generated using a peptide of the following sequence:
15 LFYNEQQEARGVHTQRMQ (SEQ ID NO:3).

Cell counting: 0.3 X10⁶ cells are plated in 60-mm dishes (GIBCO) in 5 ml medium containing 10% fetal calf serum (FCS). After overnight culture, cells are grown in serum-free medium for 24 h, and then stimulated with various concentrations
20 of IGF, IGFBP-2 and antibody to IGFBP-2 for 48 h. Control cells are incubated with control (non-immune) rabbit IgG. At term, cells will be dispersed with trypsin-EDTA solution and counted with the help of the Coulter Electronic particle counter (Model ZF, Coulter Electronics, Hialeah, FL).

25 **MTT assay:** 6,000 cells are seeded/well in 96-well plates and grown overnight in 10% FCS. Cells are maintained under serum-free conditions for 24 h, and then stimulated for 48 h with various concentrations of IGF, IGFBP-2 and antibody to IGFBP-2. The final cell number is determined with the help of MTT dye (Sigma M-2128) (Singh *et al.*, 1994). The plates are read immediately at 540 nm on a scanning
30 multi-well spectrophotometer (Model Vmax: Molecular Devices, Melno Park, CA). Optical absorbance reading from test wells is corrected for basal control absorbance values from cell-free wells containing culture media only.

Clonogenic assay: Cells are seeded at equal concentrations (8,000 cells/well) in 6-well culture plates in 0.3% agar in growth medium containing IGF, IGFBP-2 and antibody to IGFBP-2 (MacPherson and Montagnier, 1964). The colonies are stained 5 with methylene blue, and total number of colonies (> 1 mm in size)/well is counted with the help of an inverted-light microscope.

RNA Extraction: Total RNA is extracted from cells by a single-step phenol and guanidinium thiocyanate extraction method, using RNA Stat 60 (Tel-Test, Inc., 10 Friendswood, TX). Total RNA is quantitated by Spectrophotometric readings at wavelengths of 260/270/280.

RT-PCR: cDNA is prepared from 1 microgram of total RNA, using AMV reverse transcriptase (Promega). Ten percent of the cDNA prepared is used for 15 amplification by PCR, (30-32 PCR cycles), using appropriate primers. The PCR reaction is performed in 50 microliters buffer containing 2 microliters cDNA, 20 pmol of each primer, 250 uM dNTPs, and 0.3 units Taq polymerase. The reactions are denatured at 94°C for 30 sec, annealed at 60°C for 45 sec, and elongated at 72°C for 1 min/cycle. Twenty microliters of mixture from each reaction is then electrophoresed on 20 1% agarose and visualized with ethidium bromide staining.

Analysis of RNA by Northern Hybridization: About 10 micrograms of RNA per sample is electrophoresed through formaldehyde-agarose gels, transferred to nylon membranes, and cross-linked either with ultraviolet light or baking. Partial length 25 complementary probes (cDNA) are used for analysis of mRNA. The cDNA probes are labeled by nick translation, using commercial kits from Promega (Madison, WI), and then hybridized to membranes using standard conditions. Membranes are washed, briefly air-dried, exposed to X-ray film for an appropriate amount of time for the signal to develop, and the autoradiograms analyzed.

B. RESULTS

RNA samples prepared from LNCap and DU145 prostate cancer cell lines were analyzed for expression of IGFBP-2 mRNA transcripts by RT-PCR. A DNA fragment consistent with the predicted size (442 bp) was present from the cells that were 5 analyzed, demonstrating that message for IGFBP-2 is present in these cell lines.

The effect of IGFBP-2 and IGFBP-2 Ab on the growth of LNCap and DU145 prostrate cancer cells was then examined. LNCap and DU145 cells were treated with different doses of IGFBP-2 or IGFBP-2 Ab and growth was measured by counting the 10 total number of cells. Control cells are grown in 1%, 5% or 10% concentrations of Fetal Calf Serum (FCS) while experimental cells were grown under serum free conditions with the indicated additives. FIG. 1A shows the effects of either 125, 250 or 500 ng/ml IGFBP-2 or 125, 250 or 500 ng/ml IGFBP-2 antibodies on the growth of LNCap cells as compared to control cells grown in 1%, 5% or 10% FCS. FIG. 15 1B shows the effects of these same treatments on DU145 cells. While IGFBP-2 stimulated the growth of both cell lines, antibodies to IGFBP-2 inhibited the growth of both cell lines.

FIG. 1C and FIG. 1D demonstrate the effects of IGFBP-2 or antibodies to 20 IGFBP-2 in the presence or absence of IGF-I (250 ng/ml). An MTT assay was used to measure relative cell numbers. Again, antibodies to IGFBP-2 inhibited the growth of both LNCap and DU145 cells. In addition, this inhibition was still observed in the presence of exogenous IGF-1.

25 A clonogenic assay was used to test the growth of DU145 cells treated with different doses of IGFBP-2 Ab. All cultures were maintained in 10% serum and treatments were administered for 5 weeks. Colonies were stained with 1% methylene blue, and colonies were counted and their relative size determined. While IGFBP-2 treatment (250 ng/ml) resulted in larger colonies compared to control cultures, 30 culturing cells in increasing concentrations of antibodies to IGFBP-2 (125, 250 and 500 ng/ml) resulted in smaller and fewer colonies compared to control cultures.

Tumorigenic potential of DU145 cells grown in the presence of IGFBP-2 or IGFBP-2 Ab was determined in a soft agar clonogenic assay (MacPherson and Montagnier, 1964). Results of colony number are shown in FIG. 2. Cells were cultured in either 1, 5 or 10% serum alone (Serum samples, FIG. 2.) or serum free with 125, 250, or 500 ng/ml IGF-1, IGFBP-2 or antibodies to IGFBP-2. The total number of colonies/well in wells seeded with IGFBP-2 Ab was less than 1-5% of the numbers in wells seeded with IGFBP-2.

RT-PCR analysis was used to determine the relative levels of IGF-I mRNA in LNCap cells treated with IGFBP-2 Ab. RNA samples from LNCap cells growing in culture for 3 days in the presence of varying concentrations of IGFBP-2 Ab (125, 250 or 500 ng/ml) were subjected to RT-PCR for 32 cycles, using the primers specific for hIGF-I cDNA. Ethidium bromide staining of the reaction products was used to evaluate the relative density of IGF-1 PCR products from the three control cultures compared to the experimental cultures. The relative density of IGF-I message following IGFBP-2 Ab treatment was decreased approximately 50-100%, compared with the relative density of IGF-1 message in control samples.

Prostate cancer cells are treated with either IGFBP-2 or IGFBP-2 Ab and the resultant changes in the expression of IGF system components (viz. IGFs, IGF-1R, and IGFBP-2) is examined. Expression of prostate cancer specific Prostate Tumor Inducing-1 (PTI-1) gene is also examined. The inventors are measuring IGF-I RNA and PTI-1 RNA levels using RT-PCR in response to IGFBP-2 Ab treatment. They are also examining levels of IGF-II and IGF-IR by RT-PCR and Northern hybridization/RPA, as well as receptor binding assays.

The inventors examined the effect of IGFBP-2 on the growth of prostate cancer cell lines *in vitro*, and investigated the probable mechanism by which IGFBP-2 exerts its growth-modulatory effects. The results of these studies suggest that IGFBP-2 strongly stimulates the growth of androgen-sensitive (LNCap) and androgen-independent (DU145) prostatic cancer cell lines. Growth of the cells is significantly inhibited by IGFBP-2 Antibody (Ab). The probable mechanism by which IGFBP-2 Ab

inhibited growth was by down-regulating mRNA levels of IGF-I and Prostate Tumor Inducing-1 (PTI-1) genes. Since PTI-1 is a marker for prostate cancer (Shen *et al.*, 1995; Sun *et al.*, 1997), down-regulation of mRNA levels of PTI-1 indicated cancer suppression.

5

Circulatory levels of IGFBP-2 have been reported to correlate directly with the stage and severity of cancer in prostate cancer patients. Thus, circulatory levels of IGFBP-2 may be a biomarker for progression of prostate cancer. The role of IGFBP-2 in the initiation and progression of prostate cancer is not known to date. The present 10 application demonstrates that IGFBP-2 strongly stimulated the growth of prostate cancer cell lines grown in tissue culture dishes, while suppression of IGFBP-2 significantly reversed these growth effects. Treatment with IGFBP-2 Ab resulted in reduced expression of IGF-I, which is a growth stimulating factor for prostate cells. This demonstrates that growth of prostate cancer cells can be potentiated by IGFBP-2, 15 and significantly, retarded by suppression of endogenous IGFBP-2. The inventors speculate that IGFBP-2 promotes prostate cancer in the elderly by stimulating IGF-I. Normally, the serum levels of IGF-I progressively decline due to aging. Abnormal stimulation of IGF-I by high circulatory levels of IGFBP-2 could therefore induce malignant transformation and stimulate the expression of the prostate-cancer specific 20 PTI-1 gene in the prostate of older men.

EXAMPLE 2

Effects of Stable Expression of IGFB-2 or Antisense IGFB-2 in Prostate Cell Lines

25

The inventors used stably transfected clones of aggressive (DU145) and non-aggressive (LNCap) prostate cancer cell line that are over-expressing either antisense (AS) or sense (S) IGFBP-2. Growth of cells transfected with the empty vector will serve as control (C). The growth potential and clonogenic potential of stably 30 transfected S, AS and C clones of LNCap and DU145 prostate cancer cells will be measured *in vitro*. The inventors expect that S LNCap and DU145 cells will show increased clonogenic and growth potential, compared to that of the C clones, while the

clonogenic potential of AS LNCap and DU145 cells will be significantly reduced. The inventors will measure the concentrations of the IGF factors and the PTI-1 gene products at the mRNA and protein levels in the transfected cells.

5 **A. MATERIALS AND METHODS**

Vectors: The inventors are using the mammalian expression vector, pCEP4 (Invitrogen), for the studies. This vector uses the Epstein-Barr virus origin of replication, and has Epstein-Barr virus-encoded nuclear antigen-1 (EBNA-1), which allows it to replicate extra-chromosomally in the nuclei of eukaryotic cells. It has a
10 hygromycin B gene, which allows for selection in eukaryotic cells, and a pBR322 origin of replication, which allows growth and maintenance in *Escherichia coli* cells. It uses the cytomegalovirus promoter to drive expression of the insert in a constitutive manner.

15 Plasmids (pBluescript) containing either a partial length cDNA (503 bps, for constructing AS plasmids), or a full-length cDNA (for constructing S plasmids) of hIGFBP-2 are constructed using hIGFBP-2 cDNA clones as described (Shimasaki and Ling, 1991). The appropriate cDNA fragment of hIGFBP-2 is cloned in the sense (S) or anti-sense (AS) direction in pCEP4 vectors. The directional cloning of the cDNA
20 insert is confirmed by restriction mapping and DNA sequencing (Wood *et al.*, 1993). Using lipofectamine, DU145 and LNCap cells are transfected with the AS and S pCEP4 vectors, while Control (C) cells are transfected with only the pCEP4 vectors (no insert). Cells are allowed to recover for 2 days in non-selective medium.
25 Hygromycin (Sigma Chemical Co.) is then added at the optimal concentration (predetermined to be 1 mg/ml for both the cell lines). Resistant clones are selected by the limited dilution method, and at least 4-6 single-cell clones of the cell line, containing either the C, AS or S vectors, are further expanded under continued hygromycin selection. Functionality of the clones is confirmed by Western blot analysis of the conditioned media (CM).

30

Analysis of growth, clonogenic and tumorigenic potential: The inventors are analyzing the growth of non-transfected and transfected (S, AS and C) cells either

by measuring the total cell number at the end of the experimental time point or using a semiautomatic tetrazolium-based clonogenic (MTT) assay as described in Example 1. The tumorigenic potential of the cells *in vitro* are analyzed by a soft agar clonogenic assay (MacPherson and Montagnier, 1964).

5

Receptor Binding Assay for studying the expression of IGF-IR: S, AS and C clones of LNCap and DU145 cells are subcultured in 160 mm flasks and grown to subconfluence in culture medium containing 10% FCS. Cells in culture are washed with Hank's balanced salt solution (HBSS, Gibco, Grand Island, NY) containing 0.1% bovine serum albumin and 25 mmol/L HEPES (Sigma). Cells are scraped with a rubber policeman, collected in conical tissue culture polystyrene tubes, centrifuged and resuspended in HBSS at a concentration of $2-3 \times 10^6$ cells/ml. Binding assays are performed by incubating 0.5 ml aliquots of suspended cells with 0.01 nmol/L ^{125}I -IGF-I (Amersham, Arlington Heights, IL) in the presence or absence of radio-inert IGF-I, at 16°C for 6 hours, at pH 8 in a shaking water bath. At the end of the incubation, the cells are pelleted, washed with fresh ice-cold HBSS plus 0.1% BSA, and counted for ^{125}I in a gamma-counter (model 5500, Beckman, Palo Alto, CA). The binding affinity and the total binding capacity of IGF-I to prostate cancer cells is determined from a Scatchard plot of the specific binding data. The specificity of the binding of IGF-I to the prostate cancer cells will be determined from a log-dose inhibition of specific binding of radioactive IGF-I by non-radioactive IGF-I, in terms of ED₅₀ and relative binding affinity (Singh *et al.*, 1991).

B. RESULTS

25 Autoradiographs of immunoblots of CM samples from AS and C clones of LNCap cells. Serum-free CM was collected from LNCap cells following 7 days of culture. The CM samples were concentrated and loaded in each lane (equivalent to 10^7 cells) and analyzed by Western immunoblot analysis (WIB) with specific anti-IGFBP-2 antibody. Samples from several different clones were analyzed. IGFBP-2 30 (BP2) was used as a positive control for the samples. The molecular mass of the bands (35,000 kDa) was matched with that obtained from recombinant IGFBP-2.

Comparisons of IGFBP-2 signals from the different clones was used to assess the degree of overexpression under downregulation of IGFBP-2.

Growth of prostate cancer cells was examined following suppression of 5 endogenous IGFBP-2 in LNCap and DU 145 cells by transfecting them with IGFBP-2 antisense DNA. Growth of AS- clones of LNCap cells in the presence of different concentrations of serum is shown in FIG. 3. Growth was measured in terms of optical density (O.D.) by an MTT assay. Results are mean ± SEM of 8 observations with the representative clones. A direct and positive correlation between the level of expression 10 of endogenous IGFBP-2 and the growth response of the antisense cells to varying concentrations of serum was noted, demonstrating that growth of prostate cancer cells can be inhibited by suppression of endogenous IGFBP-2.

The relative levels of IGF factors and PTI-I RNA will also be examined in the 15 AS and S clones and compared to that in the C clones of LNCap and DU145 cells. Based on the strength of our preliminary studies, we expect that we will confirm a mediating role for one or more IGF related factors in the growth-potentiating effects of IGFBP-2. Then we expect to measure significantly higher levels of the indicated IGF factors in S clones, and significantly lower levels in AS clones, compared to that in C 20 clones.

EXAMPLE 3

Effects on Prostate Cancer in an *in vivo* Animal Model Using IGFBP-2 or an Antibody to IGFBP-2

25

In this example, the inventors examine the effect of IGFBP-2 and IGFBP-2 Ab treatment on the rate of spontaneous development of prostate cancer in male retired breeders of the Copenhagen strain of rats, in which spontaneous prostatic tumors are known to develop at 22 months age (Dunning, 1963).

30

A. MATERIALS AND METHODS:

Measuring circulatory levels of IGFBP-2 by RIA: IGFBP-2 immunoreactivity is measured in 50 microliter serum samples of Copenhagen rats, using rat IGFBP-2 kit (Diagnostics Systems Laboratories, Inc., Webster, TX). Immunoprecipitates obtained in the double antibody coated RIA tubes of the kit are 5 pelleted after 4 hrs of incubation at room temperature, and radioactivity is measured in a gamma counter.

Analysis of histopathological changes: The prostate glands are dissected out and fixed in Bouin's fixative. Following routine histological procedures the glands are 10 embedded in paraffin and 5 micron sections are stained with hematoxylin and eosin, mounted with permount and observed under the microscope for microanatomical changes, as described by Noble (1982).

Examination of IGFBP-2 expression by immunocytochemical methods: 15 IGFBP-2 is detected immunocytochemically on 5 micron paraffin sections, following a standardized procedure for immunocytochemistry (Chatterjee *et al.*, 1996; Fang *et al.*, 1998), and by using 3,3'-diaminobenzidine (DAB) as the chromogen. Sections are counterstained with hematoxylin, dehydrated in alcohol and mounted in Permount for observation under the light microscope. Control sections are processed in parallel with 20 the omission of primary antibodies. A rough estimate of percentage of cells positive for IGFBP-2 and an estimate of the intensity of IGFBP-2 staining is obtained by video image analysis of the cells using a video image analysis system (Nikon Inc., Torrence, CA).

25 **Protein Extraction:** Frozen prostate glands of rats of the different age groups, and tumors generated in nude mice (Example 4) are weighed and homogenized in an ice-cold NP-40 lysis buffer (3 ml of lysis buffer/gram of tissue). The lysates are centrifuged to remove cellular debris. Protein content in the cell lystate will be estimated by the method of Lowry *et al.* (1951), using a BSA-Protein Analysis Kit 30 from Pierce Laboratories (Rockford, IL).

Western Immunoblot Analysis: Standard procedures for Western immunoblotting will be applied (Singh *et al.*, 1996). Briefly, 40-60 micrograms of the whole cell lysate is subjected to SDS 10% polyacrylamide gel electrophoresis at 35 mA for 5 h. Following electrophoresis, proteins are electroblotted onto PVDF (millipore) 5 membranes (0.5) using a Trans-Blot apparatus (BioRad). The membranes are then incubated overnight at 4°C with appropriate rat primary antibodies of the IGF system, and later with the appropriate horseradish peroxidase-conjugated secondary antibody (Research Diagnostics, Inc.). IGFBP-2 is visualized on the blots by incubating with chemiluminescence detection reagents using the ECL kit (Amersham, Arlington Heights, IL) following the manufacturer's instructions. The intensity of the bands in 10 the autoradiograms is compared to detect changes in the expression of the IGF factors of interest.

RNA Extraction: Frozen prostate glands obtained from rats of different age 15 groups, and tumors generated in nude mice (Example 4) are homogenized, and total RNA extracted by a single-step phenol and guanidinium thiocyanate extraction method, using RNA Stat 60 (Tel-Test, Inc., Friendswood, TX). Total RNA is quantitated by Spectrophotometric readings at wavelengths of 260/270/280.

RT-PCR: cDNA is prepared from 1 microgram of total RNA, using AMV 20 reverse transcriptase (Promega). 10 % of the cDNA prepared is used for amplification by PCR, (30-32 PCR cycles), using appropriate primers. The PCR reaction is performed in 50 microliters buffer containing 2 microliters cDNA, 20 pmol of each primer, 250 uM dNTPs, and 0.3 units Taq polymerase. The reactions are denatured at 25 94°C for 30 sec, annealed at 60°C for 45 sec, and elongated at 72°C for 1 min/cycle. Twenty microliters of mixture from each reaction is then electrophoresed on 1% agarose and visualized with ethidium bromide staining.

Analysis of RNA by Northern Hybridization: About 10 micrograms of RNA 30 per sample is electrophoresed through formaldehyde-agarose gels, transferred to nylon membranes, and cross-linked either with ultraviolet light or baking. Partial length complementary probes (cDNA) are used for analysis of mRNA levels for IGFs, IGF-IR

or IGFBP-2. The cDNA probes are labeled by nick translation, using commercial kits from Promega (Madison, WI), and then hybridized to membranes using standard conditions. Membranes are washed, briefly air-dried, exposed to X-ray film for an appropriate amount of time for the signal to develop, and the autoradiograms analyzed.

5

B. RESULTS

The inventors are examining whether spontaneous prostatic tumors develop in male retired breeders (4,12 and 20 months old) of Copenhagen rats is effected when the rats are administered IGFBP-2 or IGFBP-2 antibody. Baseline levels of serum 10 IGFBP-2 are measured in animals of the experimental age groups. A total of eighty four rats are grouped as follows: Group I (Control) -12 rats; Group II (IGFBP-2 treated) - 36 rats; and Group III (IGFBP-2 Ab treated) - 36 rats. Osmotic pumps are used to deliver IGFBP-2 and/or IGFBP-2 Ab. Loaded Alzet minipumps with flow regulators (flow rate 2.5 l/ hr) deliver either normal physiological saline (0.9%) (Group 15 I); IGFBP-2 (Group II) or IGFBP-2 antibody (Group III), are implanted into subcutaneous pockets on the dorsal surface of the experimental animals. The daily doses of IGFBP-2 received by animals in Group II is pre-calculated at 50% (for 12 animals), 100% (for 12 animals) and 150% (for 12 animals) above the mean baseline value, and the pumps filled accordingly. Similarly, animals in Group III are 20 administered IGFBP-2 antibody at appropriately adjusted doses. Treatment will be administered for 40, 80 or 120 days. Four animals per each dose tested in Groups II and III, and 4 animals of Group I are sacrificed at each experimental time point. Animals are euthanized following exposure to carbon dioxide and quickly decapitated. Blood is collected in heparinized tubes to quantitate serum IGFBP-2 levels. The 25 prostate glands are removed, grossly checked for any abnormalities, weighed, and either processed for routine histology and immunocytochemistry, or frozen in liquid nitrogen to examine changes in the protein and mRNA levels of IGF system components (viz. IGF-I, IGF-II, IGF-IR and IGFBP-2), and PTI-1.

30

Since the Alzet minipumps to be used can effectively function for 42 days, they will be replaced every 40th day. The inventors are generating polyclonal antibodies against rat IGFBP-2, using the help of a commercial outfit (Genemed Synthesis, Inc.,

San Francisco, CA). The crude serum will be tested for the presence of rat IGFBP-2 Ab, using IGFBP-2 ELISA assays, and the titre and specificity of the Ab will be determined. The efficacy of the Ab will be tested *in vitro* on the growth potency of LNCap cells to ensure that they produce effects similar to that measured with the 5 commercially available antibody (Genemed Synthesis, Inc., San Francisco, CA).

IGFBP-2 treatment of Copenhagen rats should result in inducing pre-neoplastic/neoplastic changes in the prostate of rats at a younger age, compared to that in untreated control rats. Conversely, IGFBP-2 Ab treatment should delay the onset of 10 the spontaneous changes that are usually measured in aged Copenhagen rats (Dunning, 1963). Circulatory levels of IGFBP-2 are significantly elevated in prostate cancer patients (Ho and Baxter, 1997; Kanety *et al.*, 1993; Figueroa *et al.*, 1998). Since prostate cancer is associated with old age, elevated levels of IGFBP-2 may be involved 15 in the development of malignancy in the prostate of older men. If IGFBP-2 promotes growth of cancer cells, then IGFBP-2 Ab may prevent malignant transformations in the prostate, and act like tumor suppressors by retarding the rate of tumor development. This application demonstrates that IGF-I and IGFBP-2 may be mediating growth 20 effects in tumors, it is quite conceivable that IGFBP-2 Ab may negate tumorigenesis in much the same way as inhibitors/antibodies of IGFs and IGF-1R have been shown to for other cancers (Wang *et al.*, 1997).

EXAMPLE 4

Tumorigenic Potential in Nude Mice of Prostate Cell Lines Expressing IGFB-2 or Antisense IGFBP-2

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The inventors are testing the tumorigenic potential of aggressive (DU145) or non-aggressive (LNCap) prostate cancer cells following modulation of levels of 30 IGFBP-2 within the cells. Stably transfected DU145 and LNCap clones that over-express either the S or the AS human IGFBP-2 mRNA (Example 2) are inoculated subcutaneously on contra-lateral sides of nude (athymic) male mice (10 animals per group). Cells will be inoculated at equal concentrations, approximately $1-10 \times 10^6$ cells for DU145, and $5-10 \times 10^6$ cells for LNCap cells in 0.2 ml Hank's balanced salt

solution (HBSS), on each side. At this concentration, DU145 cells form approximately 0.5 g tumors in about 60 days in 3 month old male athymic nude mice. In the case of LNCap cells, inoculation with even 10×10^7 cells generally does not result in developing palpable tumors till 90 days of *in vivo* growth in nude mice. At term, 5 animals are euthanized by carbon dioxide exposure, and tumors removed and weighed. A small part of the tumor will be processed for routine histology, while the rest is frozen in liquid nitrogen to measure the mRNA and protein levels of the IGF factors and the PTI-1.

10 The inventors expect to measure palpable tumors in mice inoculated with the S LNCap clones, but not C LNCap clones, within 60-90 days of *in vivo* growth. An increase in the tumorigenic potential of IGFBP-2 over-expressing DU145 is also expected. Similarly, the AS DU145 clones, in which IGFBP-2 expression is suppressed, will show delay in tumor formation in nude mice, in comparison to C 15 DU145 clones. There are methods for helping ensure that LNCap cells form tumors when injected sub-dermally. LNCap cells, co-inoculated with tissue-specific mesenchymal or stromal cells (Gleave *et al.*, 1991) or Matrigel (Gleave *et al.*, 1992) develop tumors subdermally. Also, LNCap cells inoculated directly within the prostates can grow into tumors in the orthotopic environment (Gleave *et al.*, 1992). The 20 inventors will test tumorigenic growth of S LNCap cells to that of C LNCap cells in an orthotopic environment.

EXAMPLE 5

In vivo Viral Delivery of Sense or Antisense IGFBP-2

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In this example, the inventors will generate recombinant adenoviruses and retroviruses expressing sense (S) and anti-sense (AS) IGFBP-2 and deliver these to the prostate via surgical procedures, or intra-uretherally. Retroviral or adenoviral vectors expressing S or AS IGFBP-2 will be constructed (Singh *et al.*, 1999). The inventors 30 can then deliver the S and AS expressing IGFBP-2 virus to the prostatic cells *in situ*, by using an optimal titer of the infectious viral particles (approximately 10^6 pfu/ml for retroviruses, and approximately 10^8 or 10^9 pfu/ml of adenoviruses). Delivery of the

viral particles will include: 1) intra-peritoneal (i.p.) injections (in the lower abdomen), which is likely to be least toxic, but may lack efficiency; 2) orthotopic injection into the prostatic mass bilaterally, using surgical procedures; 3) intra-urethral injections at the site of the prostate with intra-urethral cannulations. This third delivery route is the
5 method of choice to deliver adenoviruses directly to the prostate. Since adenoviruses are episomally expressing vectors, periodic injections, approximately 3 months apart will be required, for long-term studies. This may cause inflammatory responses at the surgical site. Retroviruses, on the other hand, get integrated into the host DNA, and will not require repeated injections, but can only infect dividing cells, resulting in
10 extremely poor effectiveness compared to adenoviruses.

EXAMPLE 6

Promoter Activity Of IGF-I, IGF-II and IGF-1R in Prostate Cell Lines

15 Previous results indicate correlative changes between RNA and protein levels of IGF factors in response to IGFBP-2 or IGFBP-2 Ab, suggesting changes in transcriptional activity of these genes. In order to test this possibility, the inventors are examining the promoter activity of the indicated genes (IGF-I, IGF-II, IGF-1R) by using promoter-reporter assays.

20

A. MATERIALS AND METHODS:

Transient transfection of prostate cancer cells: Full length IGF-I, IGF-1R and IGF-II promoters are cloned into promoterless luciferase reporter vectors to construct the promoter-luciferase vectors. While the promoters driving the expression 25 of hIGF-I and hIGF-1R are well defined, at least 4 promoters can be potentially used to drive the expression of IGF-II (Singh *et al.*, 1998). IGF-II promoters P3 and P4 are highly active in many cancers, while P1 and P2 are relatively silent. The inventors are confirming the activity of the four P1-P4 IGF-II promoters in the DU 145 and LNCap cells to use the appropriate promoter(s) for the present studies. The promoter-luciferase vectors are transfected to LNCap and DU145 cells. All cells are co-transfected with β -gal expression vectors to correct for transfection efficiency. After transfection, cells are maintained in serum-free media, and treated with IGFBP-2 or
30

IGFBP-2 Ab. Cells are lysed 24-48 h post transfection, and luciferase and β -gal activities measured, as described below.

5 **Luciferase and β -Gal assays:** Cells are washed with PBS and lysed using the reporter lysis buffer (Promega) as per the protocols provided by the company. Cellular debris is pelleted and the supernatant saved for analysis of luciferase and β -Gal activities. Luciferase activity is measured with the Luciferase Assay system (Promega), using 20 microliters of cell extract and 100 microliters luciferase assay buffer, at room temperature. Luciferase activity is measured within 15 sec of adding the substrate, 10 luciferin, with a Turner TD-20e Luminometer (Turner Designs, Sunnyvale, CA). The β -Gal assay is performed with the β -Galactosidase Enzyme Assay System (Promega) as per the protocols provided by the company. Ninety-six well plates are used for this assay. 30 microliters of cell extracts will be mixed with 20 microliters of lx reporter buffer and 50 microliters of 2x assay buffer in each well and incubated at 37°C for 30 15 min. The reactions will be stopped by adding 150 microliters of 1M sodium carbonate. Absorbance of the samples is read at 405 nm in a Umx Kinetic Microplate reader (Molecular Devices, Menlo Park, CA). The ratio of luciferase/ β -gal readings reflects the promoter activity of the indicated genes in the AS, S and C clones of prostate cancer cells.

20

B. RESULTS

The inventors expect that the changes measured in the levels of RNA and protein in response to IGFBP-2 or IGFBP-2 Ab treatment are reflected in similar changes in the transcriptional activity of the exogenous promoter-reporter plasmid in 25 the transient transfection experiments. Taken together, these results demonstrate that IGFBP-2 can modulate the transcription of the powerful mitogenic IGF factors. The inventors studies suggest that the promoter activity of one or more IGF factors is significantly increased on treatment with IGFBP-2, and a positive correlation is established between the levels of the various IGF factors and the *in vitro* 30 growth/clonogenic potential of the AS, S and C clones of the two cell lines.

EXAMPLE 7

Quantitation of IGF-I in *in vitro* Studies

A. MATERIAL AND METHODS

The protocols and reagents described in the previous examples are applicable
5 to this example.

B. RESULTS

The results of experiments discussed in the previous Examples suggested that
mRNA levels for IGF-I were suppressed in a dose-dependent manner following
10 treatment with IGFBP-2 antibody. The level of IGF-I protein in cell lines of treated
and untreated prostate cancer cells was evaluated. The conditioned media (CM) from
the cultures of approximately 10^7 cells of the Control and treated groups was
collected, concentrated using Amicon microconcentrators (Centricon 20, W.R. Grace,
Beverly, MA), and measured for the IGF-I and IGF-II levels by using specific RIA kits
15 (Diagnostic Systems Laboratories, Webster, TX), and following the kit instructions.
The results of the assay showed low (50 ng/ml) levels of IGF-I in the Control cultures.
Treatment with IGFBP-2 antibody resulted in undetectable levels of IGF-I in the CM
(FIG. 4). These findings strongly support my preliminary observations on the
diminution of IGF-I following administration of IGFBP-2 antibody.

20

EXAMPLE 8

Effect of Modulating IGFBP-2 *in vivo* on Tumor Initiation and Progression of Tumor Growth

25 A. MATERIAL AND METHODS

Twenty-one male athymic nude mice were divided into Control (8 animals) and
Treated (13 animals) groups. Each of the Control animals were inoculated
subcutaneously with a single injection of 2×10^6 cells mixed with 12 µg non-immune
IgG in 0.1 ml HBSS on the dorsal surface. The IgG was added to the cells
30 immediately before injection. Animals of the treated group were injected with an

equivalent number of cells mixed with anti-IGFBP-2 IgG at the same concentration, (i.e. 12 µg of anti-IGFBP-2 IgG per 2 x 10⁶ cells).

B. RESULTS

5 Tumor Initiation

After 14 days of cell inoculation, palpable tumors were seen in 7 of 8 animals of the Control group. In striking contrast to this, tumor development was noted in only 1 out of 13 animals of the treated group (FIG. 5). Hence, anti-IGFBP-2 significantly retarded the initiation of DU145 tumorigenesis in male thymic nude mice.

10

Progression of tumor growth:

Tumor development was closely monitored in the animals used in the tumor initiation studies described above. Well-formed tumors were seen in most of the experimental animals after two months of inoculation with DU 145 cells. The 15 experiment was terminated at this time to compare the weight of the tumors in the two groups of animals. Thus, at autopsy, the tumors were carefully removed, freed from adjacent tissues, and weighed.

The mean weight of the tumors in the two groups are presented in FIG. 6. The 20 mean tumor weight of tumors generated from Control DU145 cells was 1.04 + 0.16 grams, while that of cells treated with anti-IGFBP-2 IgG at the time of inoculation was 0.28 + 0.08 grams. Statistical analysis of the tumor weight following student's t-test revealed that the weight of the tumors generated from wild type DU145 cells was significantly greater than those generated from anti-IGFBP-2 treated DU145 cells.

25

EXAMPLE 9**Further Studies of Progression of Tumor Growth *in vivo*****Experiment 1**5 **A. MATERIAL AND METHODS**

Twenty-one male athymic nude mice were divided into three groups. Sets of 20×10^6 DU145 aggressive prostate cancer cells were mixed with 30, 60 or 120 μg (*i.e.*, 10, 20 or 40 μl from 3 mg/ml stock) of anti-IGFBP-2 IgG. Respective groups of animals were subcutaneously inoculated on the left side of the upper arm with 2×10^6 cells in 0.1 ml PBS. The contra-lateral side was inoculated with an equal concentration of untreated DU145 cells, and served as the control. Animals were examined for tumor development after ten days of inoculation. When tumors attained a size of 1 cm across, 300 ng of IgG was administered twice a week into the tumors on the left side of tumor-bearing animals. All treatments were continued for one month. At term, the tumors were carefully dissected out, weighed, and examined for gross anatomical features.

20 Group I: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 30 μg anti-IGFBP-2 IgG on left.

25 Group II: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 60 μg anti-IGFBP-2 IgG on left.

Group III: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 120 μg anti-IGFBP-2 IgG on left.

Group IV: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 60 µg of non-immune IgG on left.

5 Group V: Inoculated with untreated cells (2×10^6 cells) on both right and left sides.

B. RESULTS

Tumors were first evident after 16 days on the right side of animals of Groups 10 I-III. There was an apparent delay of about 6 days in the appearance of the tumor on the left side of the animals, where cells were mixed with various concentrations of anti-IGFBP-2 IgG. The size of the tumors on the left side (cells mixed with anti-IGFBP-2 IgG) was significantly smaller than tumors on the right side (untreated cells) in the experimental groups of animals (FIG. 7).

15

Experiment 2

A. MATERIAL AND METHODS

In another set of experiments, ten animals were divided into two groups (Groups A and B). Animals of Group A and B were inoculated on both sides with $2 \times 20 10^6$ DU145 cells. Tumors were evident by day 16 in both groups of animals. At this time, tumors in animals of Group A were treated with 300 ng of anti-IGFBP-2 IgG twice a week for one month, while tumors of Group B were administered 0.1 ml of saline. The experiment was terminated after one month. At autopsy, the tumors were carefully removed and weighed.

25

B. RESULTS

On comparison of the weights of the tumors, it was found that tumors in Group A animals were significantly reduced, in comparison to that in sham control animals of Group B (FIG. 8). On gross anatomical examination of the tumors, necrosis was 30 evident in most of the tumors treated with anti-IGFBP-2 IgG. Our studies suggest that IGFBP-2 antibody can significantly retard the growth of prostatic tumors in nude mice.

5 All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence
10 of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be
15 within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated
5 herein by reference.

- U.S. Patent 4883,750
- U.S. Patent 4,196,265
- U.S. Patent 4,554,101
- 10 U.S. Patent 4,578,770
- U.S. Patent 4,596,792
- U.S. Patent 4,599,230
- U.S. Patent 4,599,231
- U.S. Patent 4,601,903
- 15 U.S. Patent 4,608,251
- U.S. Patent 4,683,195
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- U.S. Patent 4,800,159
- U.S. Patent 5,279,721
- 20 U.S. Patent 5,354,855
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CLAIMS:

1. A method of inhibiting an IGFBP-2 dependent cancer cell comprising reducing IGFBP-2 activity level with an IGFBP-2 modulator.
5
2. The method of claim 1, wherein inhibiting an IGFBP-2 dependent cancer cell comprises altering proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion.
10
3. The method of claim 1, wherein said modulator decreases the amount of IGFBP-2.
15
4. The method of claim 1, wherein said modulator inhibits expression of IGFBP-2.
5. The method of claim 1, wherein said modulator inhibits transcription of IGFBP-2.
20
6. The method of claim 1, wherein said modulator inhibits translation of IGFBP-2.
7. The method of claim 1, wherein said modulator inhibits secretion of IGFBP-2.
25
8. The method of claim 1, wherein said modulator is an agonist or antagonist of IGFBP-2.
9. The method of claim 1, wherein said modulator of IGFBP-2 is a nucleic acid containing a promoter operably linked to a IGFBP-2 gene segment.
30
10. The method of claim 9, wherein the IGFBP-2 gene segment is positioned, in reverse orientation, under the control of a promoter that directs expression of an antisense product.

11. The method of claim 9, wherein the nucleic acid encodes a ribozyme specific for an RNA transcript of IGFBP-2 in a cell expressing an RNA transcript of IGFBP-2.
- 5 12. The method of claim 9, wherein the nucleic acid segment is located on a vector.
13. The method of claim 12, wherein the vector is a viral vector selected from the group consisting of adenovirus, retrovirus, herpes virus, adeno-associated virus, and vaccinia virus.
- 10 14. The method of claim 1, wherein said modulator is an antibody composition comprising an antibody that recognizes IGFBP-2.
- 15 15. The method of claim 14, wherein said antibody composition comprises a monoclonal antibody.
16. The method of claim 14, wherein said antibody composition comprises polyclonal antibodies.
- 20 17. The method of claim 14, wherein said antibody is a humanized antibody.
18. The method of claim 1, wherein said modulator is at least one polypeptide that interacts with IGFBP-2.
- 25 19. The method of claim 21, wherein said polypeptide comprises single chain antibodies, IGF-I, IGF-II, IFGBP-2 receptor, or a fusion protein or fragment thereof.
20. The method of claim 1, wherein said modulator is a polypeptide involved in post-translational modification of IGFBP-2.
- 30 21. The method of claim 1, wherein said cell is in a mammal.

22. The method of claim 21, wherein said mammal is a human.

23. The method of claim 1, wherein said cell is derived from the prostate.

5 24. A method of treating a subject with cancer comprising administering to said subject an effective amount of a first IGFBP-2 modulator.

25. The method of claim 24, wherein said modulator is an antibody composition comprising an antibody that recognizes IGFBP-2.

10

26. The method of claim 25, wherein said antibody composition comprises a monoclonal antibody.

15

27. The method of claim 25, wherein said antibody composition comprises polyclonal antibodies.

28. The method of claim 25, wherein said antibody is a humanized antibody.

29. The method of claim 24, wherein said modulator is a ribozyme or anti-sense
20 sequence complementary to the IGFBP-2 gene.

30. The method of claim 24, wherein the subject is human.

31. The method of claim 24, wherein said treating a subject with cancer comprises
25 killing a cancer cell, inhibiting the growth of a cancer cell, inducing apoptosis of a
cancer cell, inhibiting metastatic potential of a cancer cell, reducing tumor burden, or
inducing tumor regression.

32. The method of claim 24, wherein the cancer is prostate cancer.

30

33. The method of claim 24, further comprising treating the subject with a second agent, wherein the second agent is a second modulator of IGFBP-2, a therapeutic

polypeptide, nucleic acid encoding a therapeutic polypeptide, a chemotherapeutic agent, or a radiotherapeutic agent.

34. A method of screening a candidate substance for anti-tumor activity comprising
5 the steps of:

- (i) contacting a first IGFBP-2 dependent cell with said candidate substance; and
- (ii) comparing one or more characteristics of said first cell in the presence
10 of said candidate substance with one or more characteristics of a second IGFBP-2 dependent cell in the absence of said candidate substance.

35. The method of claim 34, wherein said characteristic is selected from the group consisting of IGFBP-2 expression, proliferation, metastasis, contact inhibition, soft
15 agar growth, cell cycle regulation, tumor formation, tumor progression, and tumor invasion.

36. The method of claim 34, wherein said cell is contacted *in vitro*.

20 37. The method of claim 34, wherein said cell is contacted *in vivo*.

38. A pharmaceutical composition comprising a recombinant vector containing a promoter operably linked to a IGFBP-2 gene segment.

25 39. The pharmaceutical composition of claim 38, wherein the IGFBP-2 gene segment is positioned, in reverse orientation, under the control of a promoter that directs expression of an antisense product.

40. The pharmaceutical composition of claim 38, wherein the recombinant vector
30 expresses a ribozyme specific for an RNA transcript of IGFBP-2 in a cell expressing an RNA transcript of IGFBP-2.

41. A method of treating a patient with cancer comprising administering to the patient a composition comprising a peptide comprising at least 8 contiguous amino acids from SEQ ID NO:2 in an amount effective to convey a therapeutic benefit on the patient.

5

42. The method of claim 41, wherein the composition further comprises an adjuvant.

43. The method of claim 42, wherein the adjuvant and the peptide are covalently linked.

10 44. The method of claim 41, wherein the peptide comprises at least 8 contiguous amino acids from SEQ ID NO:3.

15 45. The method of claim 44, wherein the peptide comprises the amino acids of SEQ ID NO:3.

46. The method of claim 41, wherein the therapeutic benefit conveyed on the patient is inhibition of an IGFBP-2-dependent cancer cell.

20

47. The method of claim 46, wherein inhibition of an IGFBP-2 dependent cancer cell comprises altering proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion.

25

48. The method of claim 41, wherein the patient has a tumor.

49. The method of claim 41, wherein the cancer is prostate cancer.

30 50. The method of claim 49, wherein the therapeutic benefit conveyed on the patient is a reduction in tumor size.

51. The method of claim 41, wherein the patient is further administered chemotherapy, radiotherapy, surgery, hormone therapy, or gene therapy.

52. A method of preventing cancer in a subject comprising administering to the subject a composition comprising a peptide comprising at least 8 contiguous amino acids from SEQ ID NO:2 in an amount effective to elicit an immune response against IGFBP-2.

53. The method of claim 51, wherein the composition further comprises an adjuvant.

54. The method of claim 53, wherein the adjuvant is covalently linked to the peptide.

SEQUENCE LISTING

<110> CHAKRABARTY, SHILLA
SINGH, POMILA

<120> SUPPRESSION OF ENDOGENOUS IGFBP-2 TO INHIBIT CANCER

<130> UTSG233

<140> FILED HEREWITH
<141> 2000-05-17

<150> 60/134,581
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<170> PatentIn Ver. 2.1

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Val Phe Arg Glu Lys Val Thr Glu Gln His Arg Gln Met Gly Lys Gly
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Tyr Ser Leu His Ile Pro Asn Cys Asp Lys His Gly Leu Tyr Asn Leu
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Lys Gln Cys Lys Met Ser Leu Asn Gly Gln Arg Gly Glu Cys Trp Cys
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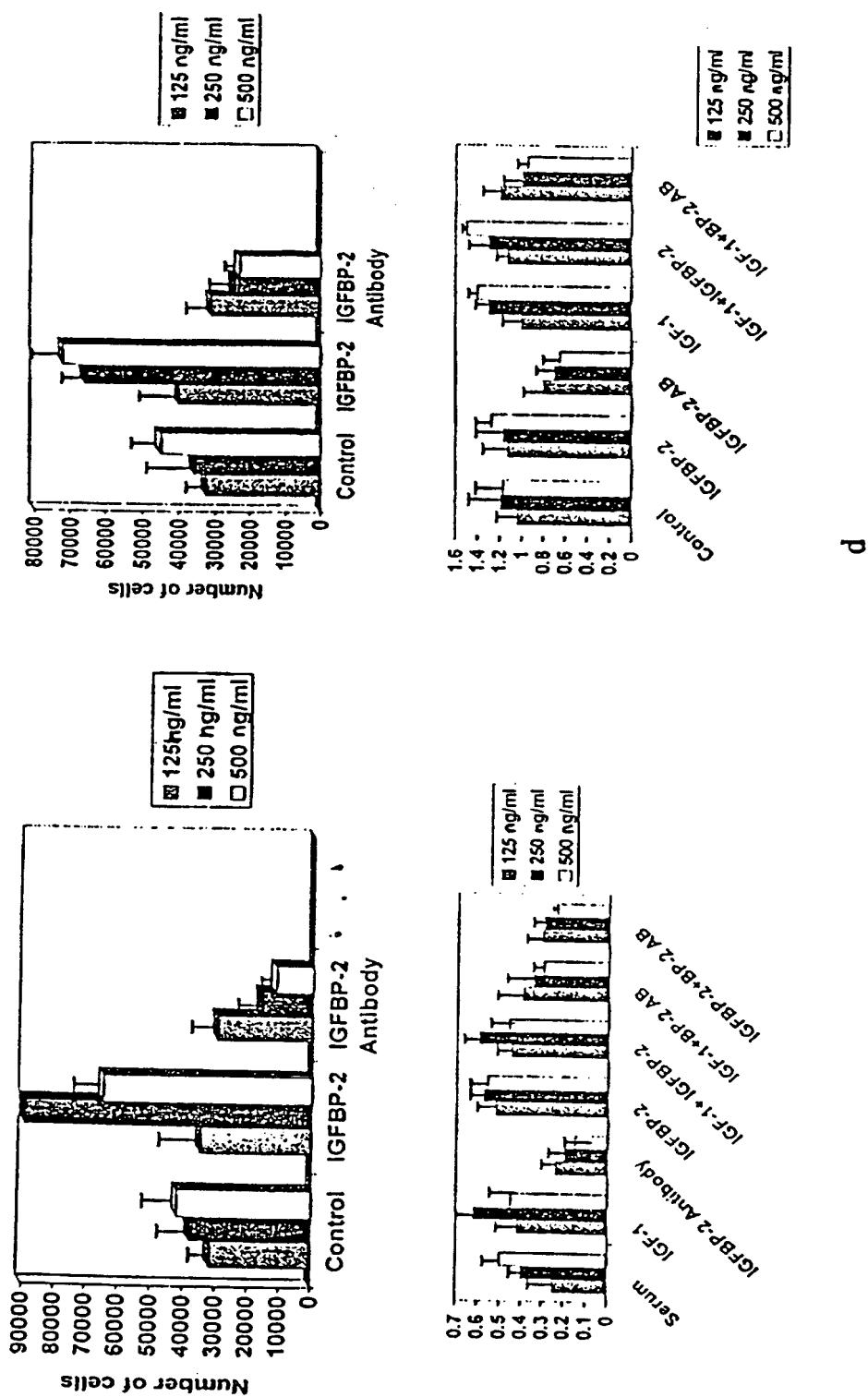


FIG. 1

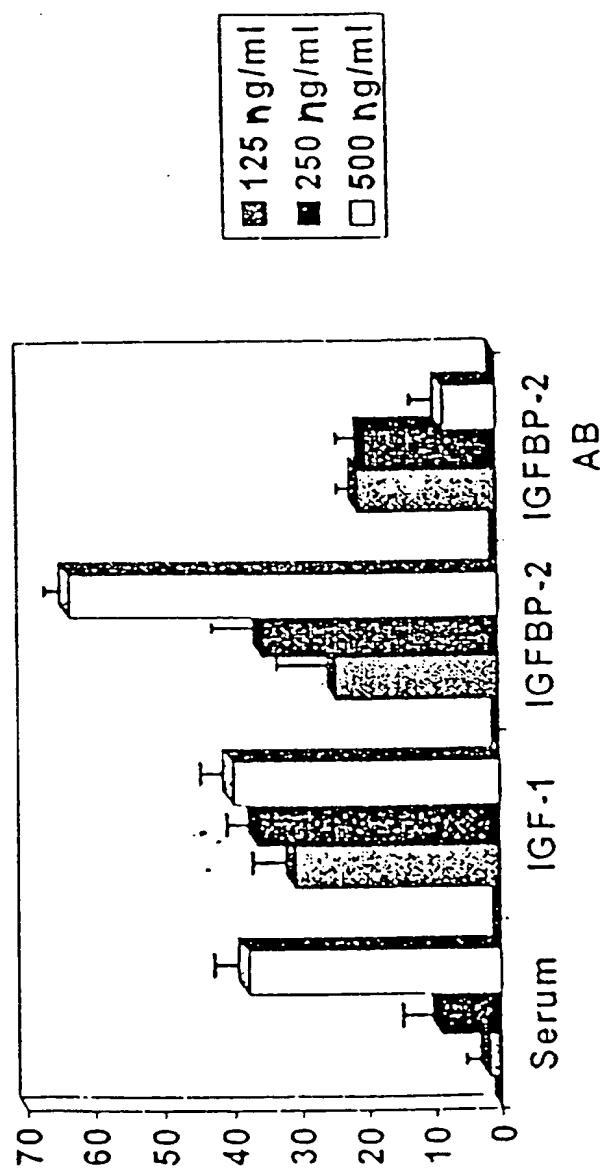


FIG. 2

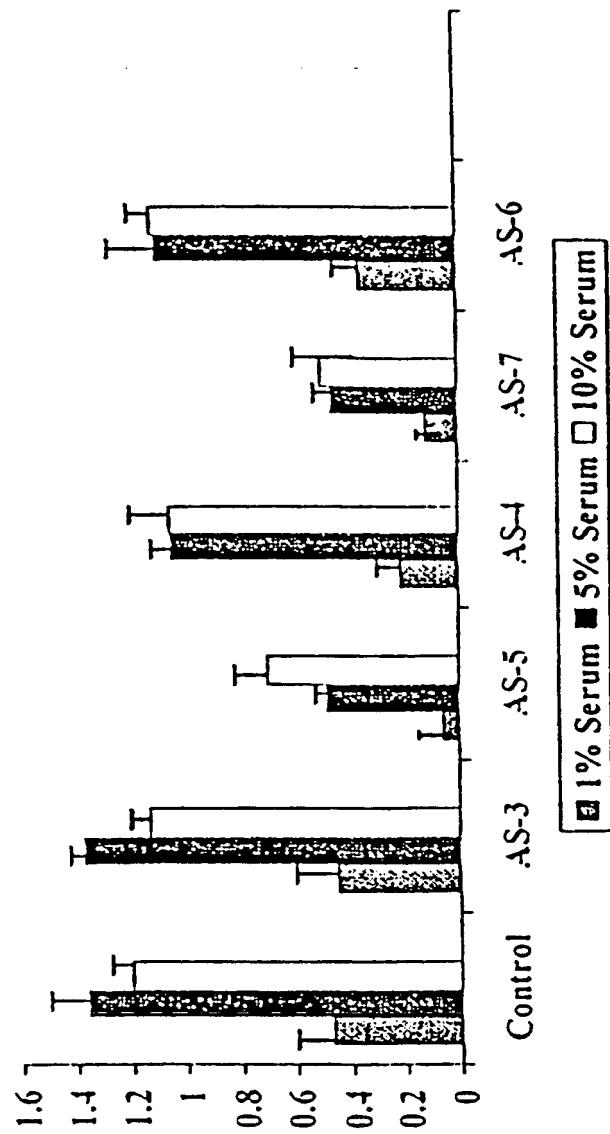


FIG. 3

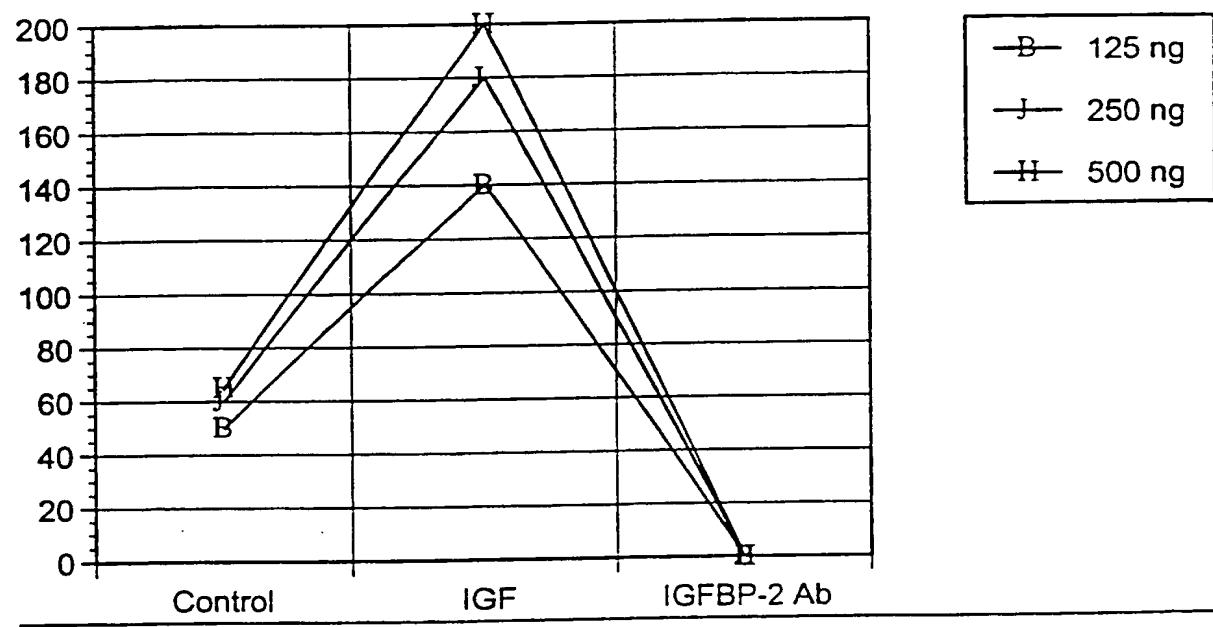


FIG. 4

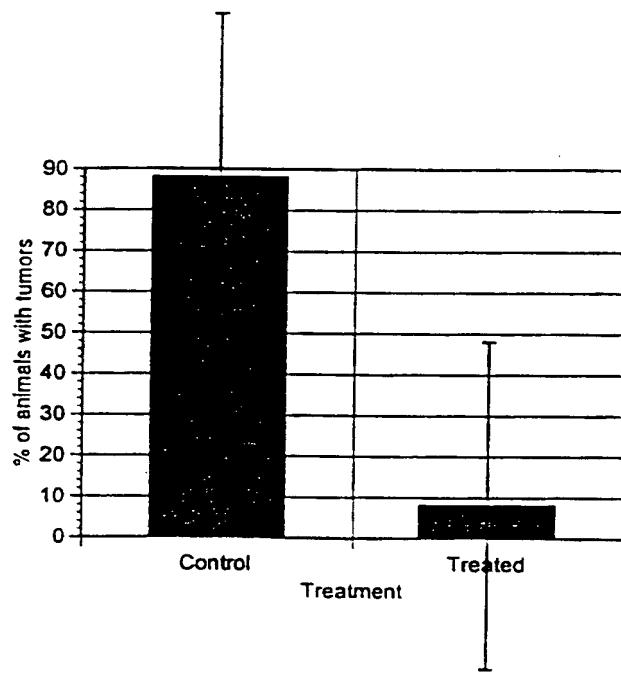


FIG. 5

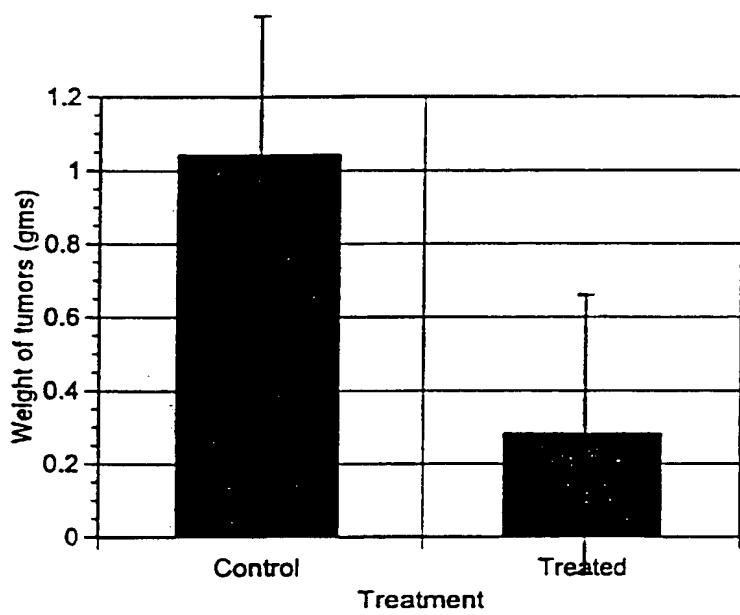


FIG. 6

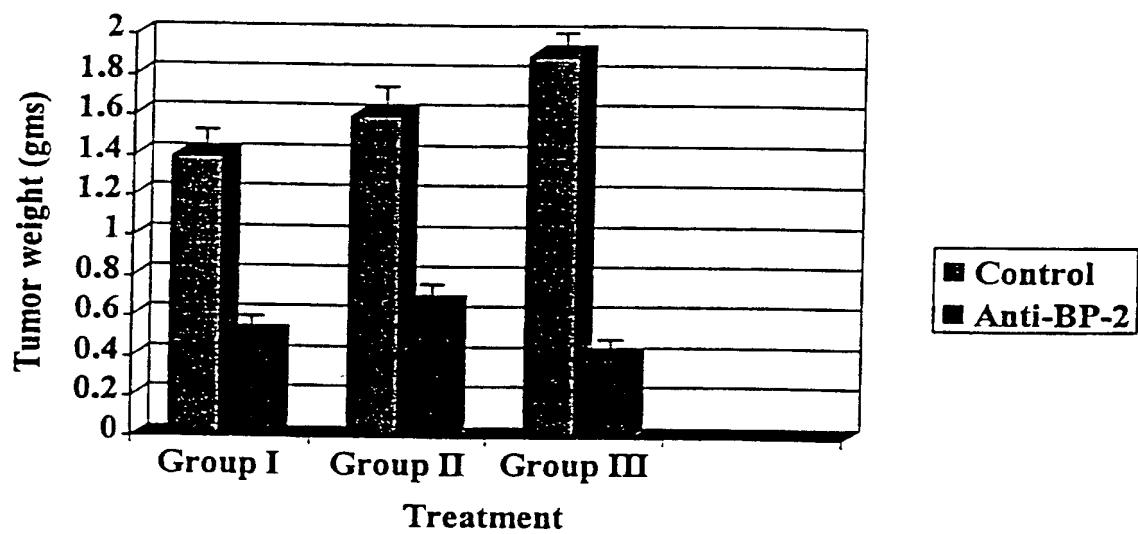


FIG. 7

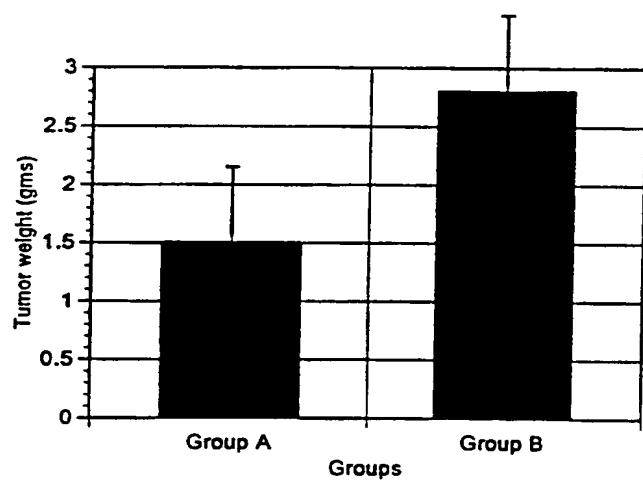


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No. PCT/US00/13574

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/17, 18; 39/00; 48/00

US CL : 514/2, 44; 424/130.1, 158.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 44; 424/130.1, 158.1, 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, BIOSIS

search terms: IGFBP-2, cancer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FORSYTH, E. Growth inhibition of a human colon cancer cell line by antisense oligonucleotides to IGFBP-2. Gastroenterology. 1995. Vol.108, No.4, suppl. page A726.	1-10, 21-22, 34-36
A	MICHELL, N.P. Insulin-like growth factor binding proteins as mediators of IGF-I effects on colon cancer cell proliferation. Growth factors. 1997. Vol. 14, No. 4, pages 269-277.	1-54
A	MICHELL, NP. Insulin-like growth factors and their binding proteins in human colonocytes: preferential degradation of insulin-like growth factor binding protein 2 in colonic cancers. British Journal of Cancer. 1997. Vol.76, No.1, pages 60-66.	1-54

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance		
"E" earlier document published on or after the international filing date	"X"	document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
22 SEPTEMBER 2000

Date of mailing of the international search report
24 OCT 2000

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Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/13574

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COHEN, P. Elevated levels of insulin-like growth factor-binding protein-2 in the serum of prostate cancer patients. <i>Journal of Clinical Endocrinology and Metabolism</i> . April 1993. Vol.76, No.4, pages 1031-1035.	1-54
A	BOULLE, N. Increased levels of insulin-like growth factor II (IGF-II) and IGF-binding protein-2 are associated with malignancy in sporadic adrenocortical tumors. <i>Journal of Clinical Endocrinology and Metabolism</i> . 1998. Vol.83, No.5, pages 1713-1720.	1-54

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**(19) World Intellectual Property Organization
International Bureau**



**(43) International Publication Date
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PCT

**(10) International Publication Number
WO 00/69454 A1**

(51) International Patent Classification⁷: A61K 38/17,
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(21) International Application Number: PCT/US00/13574

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(25) Filing Language: English

(81) Designated States (*national*): CA, JP, US.

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12 April 2001

(71) *Applicant (for all designated States except US): BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US).*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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WO 00/69454 A1

(54) Title: SUPPRESSION OF ENDOGENOUS IGFBP-2 TO INHIBIT CANCER

(57) Abstract: The present invention concerns the role of IGFBP-2 in cancer. Cancers that are dependent on IGFBP-2 would benefit from therapies targeting IGFBP-2. The invention discloses methods for inhibiting IGFBP-2 dependent cancers by modulating IGFBP-2 activity. Modulation can be achieved by decreasing the amounts of IGFBP-2 protein or IGFBP-2 activity, as well as other polypeptides that interact with or modify IGFBP-2. Preferred modulators of IGFBP-2 include antibodies specific for IGFBP-2 and antisense or ribozymes specific for IGFBP-2 nucleic acid. These modulators are useful for treating cancers derived from any tissue, with cancer of the prostate being preferred. Methods of treating a subject with cancer are disclosed as well as compositions of IGFBP-2 modulators and kits containing such modulators.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
23 November 2000 (23.11.2000)

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(10) International Publication Number
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(51) International Patent Classification⁷: **A61K 38/17,**
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77591 (US). **SINGH, Pomila** [US/US]; 3026 Acorn
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(21) International Application Number: **PCT/US00/13574**

(74) Agent: **SHISHIMA, Gina, N.**; Fulbright & Jaworski,
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78701 (US).

(22) International Filing Date: **17 May 2000 (17.05.2000)**

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(30) Priority Data:
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18 April 2002

(71) Applicant (*for all designated States except US*): **BOARD
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(US).

(15) Information about Corrections:

see PCT Gazette No. 16/2002 of 18 April 2002, Section II

Previous Correction:

see PCT Gazette No. 15/2001 of 12 April 2001, Section II

(72) Inventors; and

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(75) Inventors/Applicants (*for US only*): **CHAKRABARTY,
Shila** [IN/US]; 11013 31st Avenue N, Texas City, TX



WO 00/69454 A1

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(57) Abstract: The present invention concerns the role of IGFBP-2 in cancer. Cancers that are dependent on IGFBP-2 would benefit from therapies targeting IGFBP-2. The invention discloses methods for inhibiting IGFBP-2 dependent cancers by modulating IGFBP-2 activity. Modulation can be achieved by decreasing the amounts of IGFBP-2 protein or IGFBP-2 activity, as well as other polypeptides that interact with or modify IGFBP-2. Preferred modulators of IGFBP-2 include antibodies specific for IGFBP-2 and antisense or ribozymes specific for IGFBP-2 nucleic acid. These modulators are useful for treating cancers derived from any tissue, with cancer of the prostate being preferred. Methods of treating a subject with cancer are disclosed as well as compositions of IGFBP-2 modulators and kits containing such modulators.

DESCRIPTION**SUPPRESSION OF ENDOGENOUS IGFBP-2 TO INHIBIT CANCER****BACKGROUND OF THE INVENTION**

5 This application claims priority to U.S. Serial No. 60/134,581, which is a provisional application filed on May 17, 1999 that is hereby incorporated by reference in its entirety. The U.S. government may own rights in this invention pursuant to grant numbers CA72992 and CA60087 from the National Institutes of Health.

10 1. **FIELD OF THE INVENTION**

The present invention relates generally to the fields of biochemistry and cancer. More particularly, it concerns Insulin-like Growth Factor Binding Protein 2 (IGFBP-2) and its relevance to cancer.

15 2. **DESCRIPTION OF RELATED ART**

Prostate cancer is the most common non-skin cancer diagnosed in men in the United States. With about 184,000 new cases found, and about 39,200 deaths attributed to prostate cancer each year, it is second only to lung cancer as the most common cause of death from malignancy. The cause of prostate cancer is unknown, 20 and the tumor often remains clinically silent until it has reached an advanced stage in men over 60 years of age. The methods currently used to diagnose prostate cancer, such as physical exam, monitoring Prostate-Specific Antigen (PSA) levels, tissue biopsy and ultrasound and bone scans, are restricted in both sensitivity and specificity. Since the survival of prostate cancer patients depends on early detection and treatment, 25 novel strategies for early diagnosis and treatment of the disease must be developed.

The Insulin-like Growth Factor (IGF) system is a multi-component network of molecules (Juul *et al.*, 1995) involved in the regulation of cell growth in a variety of organs (Rajaram *et al.* 1997), including the prostate (Cohen *et al.*, 1994; Thrasher *et al.*, 1996). The IGF binding proteins (IGFBPs) are a family of high affinity binding

proteins, which regulate IGF availability to the receptors, and are now being recognized as a new class of growth modulators (Cohen and Rosenfeld, 1994). IGFBP-2 is elevated in the serum of patients with prostate cancer (Ho and Baxter, 1997; Kanety *et al.*, 1993; Figueroa *et al.*, 1998), but not in benign prostatic hyperplasia (BPH) (Ho and Baxter, 1997; Cohen *et al.*, 1993). Elevation of IGFBP-2 5 is related to the stage of the tumor and serum prostate-specific antigen (PSA) levels, suggesting a positive correlation between IGFBP-2 and prostate cancer. Increment in serum IGFBP-2 correlates positively with severity of many other cancers as well (Reeve *et al.*, 1992a and 1992b; Karasik *et al.*, 1994; Kanety *et al.*, 1996; Wang *et al.*, 10 1997; Flyvbjerg *et al.*, 1997; Menouny *et al.*, 1998), but the role of IGFBP-2 in carcinogenesis was unexplored. It is not clear whether the increase in serum IGFBP-2 seen in prostate cancer is causative of progression of the cancer or simply a passive marker.

15 Insights into improved therapies for cancers are needed. While there are available therapies for various cancers, including chemotherapy, radiotherapy, and immunotherapy, novel approaches showing improved success must be developed. Often times, it is a unique combination of therapies that results in the best efficacy towards a given cancer. In addition to the roles of growth factors and their receptors in 20 cancer, the roles of growth factor serum binding proteins in cancer needs to be addressed.

SUMMARY OF THE INVENTION

25 The present invention discloses a method of inhibiting an IGFBP-2 dependent cancer cell comprising reducing IGFBP-2 activity level with an IGFBP-2 modulator. Inhibiting an IGFBP-2 dependent cancer cell includes altering proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion.

The IGFBP-2 modulator may decrease the amount of IGFBP-2, inhibit expression, transcription, translation, secretion, or inhibit the functional activity of IGFBP-2. The IGFBP-2 modulator may be an agonist or antagonist of IGFBP-2.

5 The modulator of IGFBP-2 is a nucleic acid containing a promoter operably linked to a IGFBP-2 gene segment. In preferred embodiments, the IGFBP-2 gene segment is positioned, in reverse orientation, under the control of a promoter that directs expression of an antisense product. In other embodiments, the nucleic acid encodes a ribozyme specific for an RNA transcript of IGFBP-2 in a cell expressing an
10 RNA transcript of IGFBP-2. The promoter that is operably linked to a gene segment that encodes IGFBP-2 functions in a mammalian cell.

15 The nucleic acid segment containing a promoter operably linked to a IGFBP-2 gene segment may be located on a vector, such as a plasmid vector. In other embodiments, the vector is a viral vector selected from the group consisting of adenovirus, retrovirus, herpes virus, adeno-associated virus, and vaccinia virus. The nucleic acid may be packaged in a virus particle. The promoter that is operably linked to a gene segment that encodes IGFBP-2 functions in a mammalian cell.

20 In preferred embodiments, the IGFBP-2 modulator is an antibody composition comprising an antibody that recognizes IGFBP-2. The antibody composition may comprise a monoclonal antibody, polyclonal antibodies, or a humanized antibody.

25 The IGFBP-2 modulator may be at least one polypeptide that interacts with IGFBP-2, and may comprises IGF-I, IGF-II, IFGBP-2 receptor, or a fusion protein or fragment thereof. The IGFBP-2 modulator may also be a polypeptide involved in post-translational modification of IGFBP-2.

30 The IGFBP-2 dependent cell may be in cell culture or in an animal. The animal may be a mammal and may be a human. The IGFBP-2 dependent cell may be derived from the brain (*e.g.*, glioblastoma, astrocytoma, oligodendrogloma, ependymomas), neurofibrosarcoma, meningia, lung, liver, spleen, kidney, lymph node, pancreas, small

intestine, blood cells, colon or colorectal region, stomach, thyroid, breast, endometrium, prostate, testes, ovary, skin, head and neck, esophagus, bone marrow, blood or other tissue. In preferred embodiments, the cell is derived from the prostate.

5 Also disclosed are methods of treating a subject with cancer comprising administering to the subject an effective amount of a IGFBP-2 modulator. In preferred embodiments, the subject is a human. The IGFBP-2 modulator may be an antibody composition comprising an antibody that recognizes IGFBP-2. The antibody composition may comprises a monoclonal antibody, polyclonal antibodies, or a
10 humanized antibody. Alternatively, the modulator may be a ribozyme or antisense sequence complementary to the IGFBP-2 gene.

15 Treating a subject with cancer may result in killing a cancer cell, inhibiting the growth of a cancer cell, inducing apoptosis of a cancer cell, inhibiting metastatic potential of a cancer cell, reducing tumor burden, or inducing tumor regression. The cancer may be a cancer of the brain (e.g., glioblastoma, astrocytoma, oligodendrolioma, ependymomas), neurofibrosarcoma, meningia, lung, liver, spleen, kidney, lymph node, pancreas, small intestine, blood cells, colon or colorectal region, stomach, thyroid, breast, endometrium, prostate, testes, ovary, skin, head and neck,
20 esophagus, bone marrow, blood or other tissue. In preferred embodiments, the cancer is prostate cancer.

25 The method of treating a subject with cancer may also comprise treating the subject with a second agent, wherein the second agent is a second modulator of IGFBP-2, a therapeutic polypeptide, nucleic acid encoding a therapeutic polypeptide, a chemotherapeutic agent, or a radiotherapeutic agent.

30 Also disclosed is a method of screening a candidate substance for anti-tumor activity comprising the steps of contacting a first IGFBP-2 dependent cell with a candidate substance, and comparing one or more characteristics of the first cell in the presence of the candidate substance with one or more characteristics of a second IGFBP-2 dependent cell in the absence of the candidate substance. Characteristic that

can be examined may be selected from the group consisting of IGFBP-2 expression, proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, and tumor invasion. The IGFBP-2 dependent cell may be contacted *in vitro* or *in vivo*.

5

Other embodiments of the present invention include therapeutic and prophylactic methods to treat or prevent cancer. The cancer may involve a tumor. In some aspects, the methods of treating a cancer patient involve administering to the patient a composition that contains a peptide having at least 8 contiguous amino acids from the amino acid sequence of human IGFBP-2 (SEQ ID NO:2) in an amount effective to convey a therapeutic benefit on the patient. The term "therapeutic benefit" used throughout this application refers to anything that promotes or enhances the well-being of the subject with respect to the medical treatment of his condition, which includes treatment of cancer or other hyperproliferative diseases. A list of nonexhaustive examples of such benefit includes extension of the subject's life by any period of time, decrease or delay in the neoplastic development of the disease, decrease in hyperproliferation, reduction in tumor growth or size, delay of metastases, reduction in cancer cell or tumor cell proliferation rate, and a decrease in pain to the subject that can be attributed to the subject's condition. In the context of the invention, the therapeutic benefit conveyed on the patient may generally involve inhibition of an IGFBP-2-dependent cancer cell, whose characteristics have been discussed above.

In some aspects of the present invention, the preventative and therapeutic methods involve a composition that includes an adjuvant in addition to an IGFBP-2 peptide or polypeptide. The adjuvant may or may not be linked to the peptide or polypeptide. If the adjuvant is linked to the IGFBP-2 peptide or polypeptide, they may be covalently linked. It is contemplated that the IGFBP-2 peptide may be of any length, though the peptide of SEQ ID NO:3 has been used to generate antibodies. A peptide that contains at least 8 contiguous amino acids from SEQ ID NO:3 may be employed in the methods of the present invention, as well as a peptide comprising SEQ ID NO:3.

It is also contemplated that the therapeutic methods of the invention may be employed in combination with another anti-cancer treatment such as chemotherapy, radiotherapy, surgery, hormone therapy, or gene therapy, which may be administered before administration of the IGFBP-2 directed agent, which includes an IGFPB-2 peptide that confers a therapeutic benefit, as well as after or at the same time as the IGFBP-2 directed agent.

The preventative methods of the invention rely upon the immune system to effect a response against IGFBP-2. An immune response includes a humoral as well as 10 a cellular or cell-mediated response. Thus, it is contemplated that a subject could be immunized with an IGFBP-2 peptide or polypeptide to generate a level of humoral and/or cellular immunity against IGFBP-2. Multiple immunizations or boosters may be given to a subject to maintain the immune response.

15 Also disclosed are pharmaceutical compositions comprising a recombinant vector containing a promoter operably linked to a IGFBP-2 gene segment. The pharmaceutical composition may have the IGFBP-2 gene segment positioned, in reverse orientation, under the control of a promoter that directs expression of an antisense product. Alternatively, the pharmaceutical composition may be a 20 recombinant vector expressing a ribozyme specific for an RNA transcript of IGFBP-2 in a cell expressing an RNA transcript of IGFBP-2.

Finally, kits are disclosed comprising a suitable container and at least a first antibody composition that recognizes IGFBP-2 or a kit comprising a suitable container 25 and at least a recombinant vector containing a promoter operably linked to a nucleic acid segment that encodes IGFBP-2.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also 30 consistent with the meaning of "one or more," "at least one," and "one or more than one."

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A, FIG. 1B, FIG. 1C, and FIG. 1D. Effect of IGFBP-2 and IGFBP-2 antibody (Ab) on the growth of LNCap and DU145 prostate cancer cells. LNCap and DU145 cells were treated with different doses of IGFBP-2 or IGFBP-2 Ab, with or without IGF-I (250 ng/ml), and growth was measured by counting the total number of cells (FIG. 1A. - LNCap; FIG. 1B. - DU145), or by an MTT assay (FIG. 1C. - LNCap; FIG. 1D. - DU145). The Y axis of panels FIG. 1C. and FIG. 1D. represent Optical Density at 560 nm. In the sections designated by "Serum" and "Control," the three bars represent growth of Control cells in 1%, 5% and 10% concentrations of fetal calf serum (FCS).

FIG. 2. Tumorigenic potential of DU145 cells grown in the presence of IGFBP-2 or IGFBP-2 Ab, determined in a soft agar clonogenic assay. The total number of colonies/well in wells seeded with IGFBP-2 Ab was less than 1-5% of the numbers in wells seeded with IGFBP-2.

FIG. 3. Growth of AS-clones of LNCap cells in the presence of different concentrations of serum. Growth was measured in terms of optical density

(O.D.) by an MTT assay. Results are mean \pm SEM of 8 observations with the representative clones.

5 **FIG. 4.** IGF-I protein levels in CM collected from Control, IGF and IGFBP-2 treated cultures of DU145 cells. The levels of IGF increase 3-4 fold times following treatment with IGF. However, the levels of IGF are undetectable following treatment with IGFBP-2 antibody.

10 **FIG. 5.** Initiation of DU145-generated tumors in nude mice following inoculation with cells mixed with non-immune IgG (Control) and cells mixed with anti-IGFBP-2 IgG (Treated). The initiation of tumors was delayed significantly ($p<0.001$) when cells are co-inoculated with anti-IGFBP-2.

15 **FIG. 6.** Weight comparison of tumors generated in male athymic nude mice from wild type (WT) and anti-IGFBP-2 IgG mixed cells (Cells + IGFBP-2 antibody ("BP-2 Ab"))). Tumors generated from WT cells are significantly ($p<0.001$) larger than those generated from cells mixed with anti-IGFBP-2 IgG.

20 **FIG. 7.** Effect of anti-IGFBP-2 IgG on the growth of DU145 prostatic tumors as measured by tumor weight. Group I: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 30 μg anti-IGFBP-2 IgG on left. Group II: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 60 μg anti-IGFBP-2 IgG on left. Group III: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 120 μg anti-IGFBP-2 IgG on left.

30 **FIG. 8.** Effect of anti-IGFBP-2 IgG on total tumor weight in control and treated groups of animals. Animals of Group A were inoculated on the left side with 2×10^6 DU145 cells mixed with 12 μg of non-immune IgG, and with an equal

concentration of untreated cells on the right side. Group B animals were inoculated on both sides with untreated DU145 cells.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

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The present invention concerns the role of IGFBP-2 in cancer. Cancers that are dependent on IGFBP-2 would benefit from therapies targeting IGFBP-2. The invention discloses methods for inhibiting IGFBP-2 dependent cancers by modulating IGFBP-2 activity. Modulation can be achieved by decreasing the amounts of IGFBP-2 protein or IGFBP-2 activity, as well as other polypeptides that interact with or modify IGFBP-2. Preferred modulators of IGFBP-2 include antibodies specific for IGFBP-2 and antisense or ribozymes specific for IGFBP-2 nucleic acid. These modulators are useful for treating cancers derived from any tissue, with cancer of the prostate being preferred. Methods of treating a subject with cancer are disclosed as well as compositions of IGFBP-2 modulators and kits containing such modulators.

10

The present invention is directed at methods for reducing IGFBP-2 activity in such a way as to affect an IGFBP-2 dependent cell. The effect on the IGFBP-2 dependent cell can be an alteration in proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion. IGFBP-2 activity can be reduced *in vitro* or *in vivo* by a number of methods as detailed below.

15

Three general approaches are described for decreasing the amount of IGFBP-2 activity. These are inhibiting IGFBP-2 synthesis, inactivating IGFBP-2, or by increasing the breakdown or clearance of IGFBP-2. These three approaches and methods for achieving them are detailed below.

20

Inhibiting the synthesis is done in cells that are actively synthesizing IGFBP-2. IGFBP-2 may be synthesized by the IGFBP-2 dependent cell, where the IGFBP-2 would be acting in an autocrine manner on the same cell. Alternatively, IGFBP-2 synthesis may be by a second cell distinct from the IGFBP-2 dependent cell in an

endocrine manner. Strategies for inhibiting the synthesis of IGFBP-2 can target transcription of the gene encoding IGFBP-2, target IGFBP-2 messenger RNA, target translation of the IGFBP-2 message, or target secretion of IGFBP-2.

5 Several methods are contemplated for inhibiting IGFBP-2 transcription. The promoter for IGFBP-2 has been isolated and the inventors describe assays for determining the activity of this promoter in a given cell. Methods for inhibiting transcription include synthesis of antisense RNA complementary to the IGFBP-2 nucleic acid sequence. Antisense RNA can interact with genomic DNA, forming
10 heteroduplexes that inhibit transcription of the DNA sequence. Alternative methods could include targeting transcription factors or other accessory factors involved in transcription. Methods for introducing genetic constructs into IGFBP-2 producing cells are described, as are methods for determining IGFBP-2 expression and screens for candidate compounds that affect IGFBP-2 transcription.

15

An alternative approach for inhibiting IGFBP-2 is to target the IGFBP-2 messenger RNA. Inhibition of IGFBP-2 synthesis could either be brought about by decreasing the amount of IGFBP-2 mRNA or inhibiting its translation into IGFBP-2 polypeptide. Methods for achieving this include producing antisense IGFBP-2 RNA
20 that forms a duplex with IGFBP-2 mRNA or producing IGFBP-2 specific ribozymes. Again, methods for introducing genetic constructs into IGFBP-2 producing cells are described, as are methods for determining IGFBP-2 expression and screens for candidate compounds that effect IGFBP-2 translation.

25

IGFBP-2 is a secreted protein and methods for inhibiting its secretion would decrease IGFBP-2 activity. Cells secrete proteins through a secretory pathway, where proteins destined for secretion are targeted to the endoplasmic reticulum. These proteins then pass on to the golgi apparatus and then to the plasma membrane where they are ultimately released. One general method of inhibiting IGFBP-2 secretion is
30 based on producing polypeptides that would interact with IGFBP-2 inside the cell and interfere with its secretion. Such polypeptides include antibodies with specificity to IGFBP-2, with single chain antibodies described as one approach. Alternatively,

several polypeptides are known to interact with IGFBP-2, including IGF-I, IGF-II, and IGFBP receptors. Cosynthesis of such polypeptides, or fragments or fusion proteins containing such polypeptides, could interfere with the secretion of IGFBP-2.

5 Polypeptides such as those described above can also be used to bind and reduce IGFBP-2 activity after it has been secreted from the cell synthesizing it. Methods for using polypeptides that interact with IGFBP-2, including IGF-I, IGF-II, and IGFBP receptors, or fragments or fusion proteins containing such polypeptides are anticipated. In addition, antibodies specific for IGFBP-2 can be used to bind and inactivate IGFBP-2. Such antibodies can be monoclonal or polyclonal as well as human or humanized antibodies. Polypeptides such as these that bind IGFBP-2 can lead to its inactivation, increased clearance rate or shorter half-life, all of which will decrease overall IGFBP-2 activity.

10 15 A final method contemplated for reducing IGFBP-2 activity is to effect post-translational modifications that affect its activity. Post-translational modifications that can be targeted include phosphorylation, proteolysis and glycosylation, as well as polypeptides involved in these modifications, including kinases, phosphatases and proteases.

20 25 IGFBP-2 is a major prostatic binding protein and is dramatically elevated in serum of all patients with prostate cancer, but not in non-cancerous benign prostatic hyperplasia; the degree of elevation is directly related to the severity of the cancer. This suggests that IGFBP-2 may be involved in the development and/or progression of prostate cancer. However, the role of IGFBP-2 in prostatic carcinogenesis was previously not known. An investigation was undertaken to determine the effects of IGFBP-2 and IGFBP-2 antibody on the growth of androgen-sensitive LNCap and androgen-independent DU145 prostate cancer cells in the presence or absence of IGF-I. The inventors' results demonstrate that the growth of these cell lines was both significantly potentiated by IGFBP-2 and significantly retarded by either IGFBP-2 antibody or suppression of endogenous IGFBP-2 in transfection studies. In addition to providing a method for producing therapeutic compounds, these findings have

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profound clinical implications in the diagnosis and treatment of prostate cancer. IGFBP-2 antibody and endogenous suppression of IGFBP-2 can inhibit the growth of prostate tumors *in vitro*, then: (1) circulatory levels of IGFBP-2 can serve as a biomarker for prostatic carcinoma, and (2) suppression of IGFBP-2 by various modes 5 of action can be used as a novel strategy for treating prostate cancer.

1. INSULIN-LIKE GROWTH FACTOR AND BINDING PROTEINS

Insulin-like Growth Factors (IGFs) are low molecular weight polypeptide hormones with structural homology to proinsulin. Two different IGFs are known, 10 namely IGF-I and IGF-II, which are mitogenic *in vitro* for a wide variety of cells in tissue culture. A general review of the Insulin-like Growth Factors is given in Jones and Clemmons (1995), incorporated herein by reference. Both IGFs stimulate *in vitro* the growth of various tissues and in particular they induce collagen synthesis. IGF-I mediates the growth promoting effect of growth hormone in chondrogenesis and bone 15 formation and is therefore essential for normal growth of an organism. This is demonstrated by the fact that pygmies and toy poodles are deficient in IGF-I, but have normal growth hormone level in their serum. IGF-II is believed to play a key role in fetal development and nerve growth.

20 In addition to their primary effect on skeletal tissue, IGF-I and IGF-II also exhibit growth-stimulating functions on other tissues. Wound fibroblasts are known to produce IGFs that are effective in stimulating fibroblasts to grow and synthesize collagen, a structural protein normally required for wound healing. Vascularization of the wound tissue also is induced. Further, it has also been found that IGFs have an 25 erythropoietin-like activity in that they induce hematopoiesis.

Recent studies also have demonstrated that IGFs produced by certain cancer cells, *e.g.*, breast and kidney cancer cells, auto-stimulate the proliferation of cancer 30 cells and the vascular and fibrous tissues required to support the growth of cancer tissues.

In addition to this, both IGFs show a spectrum of metabolic activities similar to those of insulin, in that they stimulate, in particular, the transport and metabolism of glucose. The biological effects of IGFs and insulin are mediated through their binding to specific receptors. In particular, both IGFs have the ability to bind to the insulin receptor with approximately 100-fold lower affinity than does insulin.

Both IGFs have a concentration in blood approximately a hundred-fold higher than that of insulin. Hypoglycemia is prevented by a regulatory mechanism that involves carrier proteins present in blood and able to form complexes with IGFs. Thus, 10 IGFs circulate in the blood in the form of a complex that has no insulin-like activity. Through their association with carrier protein (hereinafter referred to as IGF-binding proteins or IGFBPs), binding of IGFs to cell surface receptors is inhibited. It also has been demonstrated that another function of the IGF-binding proteins is to increase the short half-life of IGFs, which are subjected to rapid proteolytic degradation when 15 present in the free form in blood.

A. INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

In the circulation, in other body fluids, and in media conditioned by cultured cells, the somatomedins (IGF-I and IGF-II) are bound to specific high-affinity carrier 20 proteins that have been implicated as modulators of IGF actions. The history of IGF binding proteins (BPs) dates back to 1984 when the existence of specific somatomedin carrier proteins in serum was first shown (Hintz, 1984). Four distinct IGFBPs have now been cloned and sequenced, and in addition, several other, not yet thoroughly characterized, BP species have been identified in various tissues (Baxter and Martin, 1989; Roghani *et al.*, 1989; Bautista *et al.*, 1990). On the basis of the sequences it became evident that many of the previously recognized BPs, known by different names, were in fact the same, falling into a defined number of classes of cloned BPs. To clarify the status of these BPs, the Workshop on IGF Binding Proteins, held in Vancouver, Canada, June 1989, proposed the names IGFBP-1, IGFBP-2, and IGFBP- 25 30 for the binding proteins with defined sequences (Ballard *et al.*, 1989). The consensus at the Workshop was that other incompletely characterized IGFBPs be referred to by

size and origin until sequenced. Since that time, several other IGFBPs, namely IGFBP-4, IGFBP-5 and IGFBP-6 have been sequenced, as described below.

Various review articles report that despite increasing interest in IGFBPs in recent years, their functions are still poorly understood (Baxter, 1988; Jones and Clemons, 1995). Baxter points to some evidence that association with BPs may not always inhibit the activity of the IGFs and that cell types producing the BPs might be able to enhance their IGF responsiveness in an autocrine fashion. Examples cited are that (1) some high molecular weight complexes from human plasma retain biological activity in rat adipocyte assays for insulin-like activity; (2) cultured human fibroblasts secrete a BP of 35 kD that increases cell IGF binding; and, (3) a pure preparation of amniotic fluid BP significantly potentiates the effect of IGF-I in stimulating DNA synthesis in porcine smooth muscle cells and fibroblasts from various species. Furthermore, it has been shown that IGFBP-3 blocks the hypoglycemic action of IGF-I when administered subcutaneously together with the IGF-I in a 1:1 ratio (Spencer *et al.*, 1991).

Another view is that IGFBPs are produced locally in all tissues to concentrate locally produced IGF-I near cells requiring the IGF-I, reducing the active role of IGF-I bound to BPs and IGF-I circulating in the blood (Isaksson *et al.*, 1987). It has been reported, for example, that IGF-I is produced locally in bone by GH (Nilsson *et al.*, 1986), and GH receptors have been found on chondrocytes (Nilsson *et al.*, 1989).

Four major functions have been proposed for IGFBPs in regulating the activities of IGFs. These are 1) to act as transport proteins in plasma and to modulate the location and movement of bound IGFs; 2) to modulate the half-life and clearance rates of IGFs; 3) to provide a means of tissue- and cell-type specific localization of IGFs; and 4) to directly modulate interactions between IGFs and IGF receptors and the resulting effects of receptor binding. However, evidence also exists for more direct biologic functions of IGFBPs, including direct effects of IGFBPs on cell function in the absence of IGFs and specific cellular receptors for IGFBPs distinct from IGF receptors.

Amniotic fluid was the first source from which IGFBP-1 was detected (Chochinov *et al.*, 1977). The protein has been purified also from tissue extract of fetal and maternal placenta and named placental protein (Kiostinen *et al.*, 1986). The 5 mature protein contains 234 amino acids, predicting a molecular mass of 25.3 kD (Lee *et al.*, 1988; WO 89/09792. IGFBP-1 migrates on SDS-PAGE at 28-35 kD depending on the stage of reduction. IGFBP-1 is a minor binding protein in serum and contains the unsaturated serum IGF-binding sites. Serum levels are inversely dependent on insulin and have a marked diurnal variation, the levels being highest early in the 10 morning. These levels increase in pregnancy up to several hundred ug/L, and amniotic fluid levels are up to 1000-fold higher than those in serum.

Carrier proteins of the IGFBP-2 class have been isolated from human fetal liver and rat and bovine cell lines (Binkert *et al.*, 1989; Rosenfeld *et al.*, 1990). In humans, 15 the mature form contains 289 amino acids and has an apparent molecular mass of 31-40 kD, depending on the stage of reduction on SDS-PAGE. In humans high IGFBP-2 levels have been found in the cerebrospinal fluid. The abundance of this protein in fetal tissue suggests that it has a role in regulating development. IGFBP-2 preferentially binds IGF-II. The nucleic acid sequence of the cDNA encoding human IGFBP-2 is 20 shown as SEQ ID NO:1 and the deduced amino acid sequence of human IGFBP-2 is shown as SEQ ID NO:2.

The majority of serum IGFs are bound to a BP composed of two parts forming a complex of molecular mass 125-150 kD. IGFBP-3 is the IGF binding subunit (β -subunit) in this complex (Baxter and Martin, 1989). It is an acid-stable glycoprotein appearing on SDS-PAGE as a major and minor band, corresponding to molecular weights of 53 kD and 47 kD, respectively. The other components in the complex are the acid-labile, non-IGF-binding subunit (α -subunit) with a molecular mass of 84-86 kD (Baxter, WO 90/0569), and IGF-I or IGF-II (γ -subunit). Sequencing of the cloned 25 cDNA for IGFBP-3 (previously known as IGFBP-53) predicts a molecular mass of 30 28.7 kD for the non-glycosylated protein and reveals that IGFBP-3 shares 33%

sequence identity with IGFBP-1 (Wood *et al.*, 1988, WO 89/09268 published Oct. 5, 1989).

A 25-kD IGFBP-4 has been isolated from cultured human osteoblast-like TE89
5 osteosarcoma cell conditioned media and sequenced (Mohan *et al.*, 1989). A similar, if not identical, IGFBP was isolated from human prostatic carcinoma cells and sequenced (Perkel *et al.*, 1990). Another similar IGFBP was identified in adult rat serum (Shimonaka *et al.*, 1989).

10 IGFBP-5 is a 252 amino acid protein that is highly conserved between species (White *et al.*, 1996). Among the IGFBPs produced IGFBP-5 is unique with regard to its marked affinity to matrix of normal bone cells, its short half-life when released, and its stimulatory effects on DNA synthesis (Schmid *et al.*, 1995). Extracellular matrix binding of IGFBP-5 leads to a decrease in its affinity for insulin-like growth factor-I
15 (IGF-I), which allows IGF-I to better equilibrate with IGF receptors (Parker *et al.*, 1998).

20 In a myoblast system, IGFBP-5 stimulated all aspects of the myogenic response to IGF-I: the later rise in myogenin mRNA, the elevation of creatine kinase activity, and the fusion of myoblasts into myotubes. In contrast, when IGFBP-5 was added in the presence of IGF-II, it inhibited both proliferation and differentiation (Ewton *et al.* 1998). This was seen in other studies, where IGFBP-5 inhibits muscle differentiation, implying a role for IGFBP-5 in regulating IGF action during myogenic development *in vivo* (James *et al.*, 1996).

25

IGFBP-6 has only recently been isolated and cloned. It is expressed during mouse development in a number of tissues, including brain (Putzer *et al.*, 1998). The effects of IGFBP-6 on neuroblastoma cell growth has been studied. (Grellier *et al.*, 1998; Babajko *et al.*, 1997) These findings suggest that IGFBP-6 contributes as an
30 autocrine inhibitor in the regulation of growth by the IGF system in these neuroblastoma cells

In other studies on IGFBP-6 actions, IGFBP-6 completely inhibited IGF-II-induced [³H]thymidine incorporation in MC3T3-E1 mouse osteoblast cells, while it had only minimal inhibitory effects on IGF-I-induced [³H]thymidine incorporation. This differential effect is associated with the fact that IGFBP-6 has greater affinity for IGF-II than IGF-I (Srinivasan *et al.*, 1996).

The levels of IGFBP in adult serum have been found to reflect the growth hormone (GH) status of individuals who are either GH-deficient or acromegalic. Thus, high levels of IGFBP-3 correlate with high levels of GH (Martin and Baxter, 1985).

Under normal conditions about 95-98% of the IGF-I in human plasma is bound to the IGFBPs. Studies on size-fractionated human serum, subjected to IGF-I RIA after extraction of each fraction to remove binding activity, have indicated that 72% of the endogenous peptide is associated with the 150-kD fraction and 25% with the 50-kD fraction (Daughaday *et al.*, 1982).

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B. IGFBP-2 ACTIVITY

The specific actions of IGFBP-2 and IGFs are unclear. Purified IGFBP-2 has been shown to inhibit [³H] thymidine incorporation into DNA in two different cell systems (Rechler and Nissley, 1990; Knauer and Smith, 1980). Inhibition of DNA synthesis was also seen using purified human IGFBP-2 in a human lung carcinoma line (Reeve *et al.*, 1993). One explanation for the observed inhibitory actions is that IGFBP-2 inhibited binding of IGFs to IGF cell surface receptors (Ross *et al.*, 1989). However, IGFBP-2 has been shown to be a weak potentiator of IGF action as well. IGFBP-2 was shown to enhance the effect of IGF-I in microvascular endothelial cells in serum-free medium (Bar *et al.*, 1989). Also, in aortic smooth muscle cells, IGFBP-2 was shown to potentiate the actions of IGF (Bourner *et al.*, 1992).

Experimental results describing actions of of IGFBP-2, and IGFBPs in general, do have to be interpreted with caution, since the extent of post-translational modification of the various IGFBPs is still under investigation. IGFBPs are known to be modified by phosphorylation (Jones *et al.*, 1991 and 1992; Mukku and Chu, 1990) and glycosylation. Proteolysis by calcium dependent serine proteases in serum or other

extracellular locations also occurs and can alter biologic activity. Finally, different target cells will have give different and often times contradictory results with respect to evaluating the biologic properties of a given IGFBP. This can most certainly be due to the identification of specific receptors or binding sites for IGFBPs.

5

As used herein, "IGFBP" refers to a protein that binds IGFs in the circulation, in other body fluids, and in media conditioned by cultured cells, as defined in the Workshop on IGF Binding Proteins held in Vancouver, Canada in June 1989 discussed above and reported in Ballard *et al.* (1989). This term includes IGFBP-1, IGFBP-2, 10 IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, and other as yet unidentified IGFBPs that have the characteristics common to all the known IGF binding proteins. The term includes animal equivalents to human IGFBPs as well as human IGFBPs, for example, the bovine, ovine, murine, porcine, and equine species. It may be from any source, whether natural, synthetic, or recombinant, provided that it will bind to the appropriate 15 binding domain of IGFs.

The term "IGFBP-2 dependent cancer cell" or "IGFBP-2 dependent cell" refers to cells whose biologic activity is modulated by IGFBP-2. Specific biologic properties or cell functions contemplated in this application include altering proliferation, 20 metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion. Other described functions of IGFBP-2 may include modulating chemotaxis, chemokinesis, immune response, and amino acid and glucose uptake. These responses may be mediated directly by IGFBP-2 or by IGFBP-2's modulation of IGF function as described above. Cells other than 25 cancer that are possibly affected by IGFBP-2 include fibroblasts, smooth muscle cells, chondrocytes, osteoblasts, keratinocytes, skeletal muscle cells, neuronal cells, mammary epithelial cells, mesangial cells, thyroid follicular cells, erythroid progenitor cells, thymic epithelium, oocytes, granulosa cells, spermatogonia, Sertoli cells and prostate cells.

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2. ANTIBODIES TO IGFBP-2 POLYPEPTIDES AND PEPTIDES

Another embodiment of the present invention is a monoclonal antibody and more preferably a human monoclonal antibody immunoreactive with the peptide sequence designated herein as SEQ ID NO:2. It is understood that antibodies can be used for inhibiting an IGFBP-2 dependent cancer cell by binding or modulating IGFBP-2. It is also understood that this antibody is useful for screening samples from human patients for the purpose of detecting IGFBP-2 present in the samples. The antibody also may be useful in the screening of expressed DNA segments or peptides and proteins for the discovery of related antigenic sequences. In addition, the antibody may be useful in passive immunotherapy for cancer. All such uses of the said antibody and any antigens or epitopic sequences so discovered fall within the scope of the present invention.

A. ANTIBODY GENERATION

In certain embodiments, the present invention provides antibodies that bind with high specificity to the IGFBP-2 polypeptides provided herein. Thus, antibodies that bind to the protein products of the isolated nucleic acid sequences of SEQ ID NO:1 are provided. As detailed above, in addition to antibodies generated against the full length proteins, antibodies also may be generated in response to smaller constructs comprising epitopic core regions, including wild-type and mutant epitopes.

20

As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE. Generally, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting.

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Monoclonal antibodies (MAbs) are recognized to have certain advantages, e.g., reproducibility and large-scale production, and their use is generally preferred. The invention thus provides monoclonal antibodies of the human, murine, monkey, rat, hamster, rabbit and even chicken origin. Due to the ease of preparation and ready availability of reagents, murine monoclonal antibodies will often be preferred.

However, "humanized" antibodies are also contemplated, as are chimeric antibodies from mouse, rat, or other species, bearing human constant and/or variable region domains, bispecific antibodies, recombinant and engineered antibodies and fragments thereof. Methods for the development of antibodies that are 5 "custom-tailored" to the patient's disease are likewise known and such custom-tailored antibodies are also contemplated.

The term "antibody" is used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, F(ab')₂, 10 single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, e.g., Harlow and Lane, "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

15

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic IGFBP-2 polypeptide composition in accordance with the present invention and collecting 20 antisera from that immunized animal.

A wide range of animal species can be used for the production of antisera. Typically the animal used for production of antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of 25 rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. 30 Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin also can be used as carriers. Means for conjugating a

polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

5 As also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Suitable molecule adjuvants include all acceptable immunostimulatory compounds, such as cytokines, toxins or synthetic compositions.

10 Adjuvants that may be used include IL-1, IL-2, IL-4, IL-7, IL-12, γ -interferon, GM-CSF, BCG, aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion also is contemplated. MHC antigens may even be used. Exemplary, often preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

15 20 In addition to adjuvants, it may be desirable to coadminister biologic response modifiers (BRM), which have been shown to upregulate T cell immunity or downregulate suppressor cell activity. Such BRMs include, but are not limited to, Cimetidine (CIM; 1200 mg/d) (Smith/Kline, PA); low-dose Cyclophosphamide (CYP; 300 mg/m²) (Johnson/ Mead, NJ), cytokines such as γ -interferon, IL-2, or IL-12 or genes encoding proteins involved in immune helper functions, such as B-7.

25 30 The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The

production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization.

A second, booster injection also may be given. The process of boosting and
5 titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

For production of rabbit polyclonal antibodies, the animal can be bled through
10 an ear vein or alternatively by cardiac puncture. The removed blood is allowed to coagulate and then centrifuged to separate serum components from whole cells and blood clots. The serum may be used as is for various applications or else the desired antibody fraction may be purified by well-known methods, such as affinity chromatography using another antibody, a peptide bound to a solid matrix, or by using,
15 e.g., protein A or protein G chromatography.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected
20 immunogen composition, e.g., a purified or partially purified IGFBP-2 polypeptide, peptide or domain, be it a wild-type or mutant composition. The immunizing composition is administered in a manner effective to stimulate antibody producing cells.

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep or frog cells also is possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred as this is most
30 routinely used and generally gives a higher percentage of stable fusions.

The animals are injected with antigen, generally as described above. The antigen may be coupled to carrier molecules such as keyhole limpet hemocyanin if necessary. The antigen would typically be mixed with adjuvant, such as Freund's complete or incomplete adjuvant. Booster injections with the same antigen would 5 occur at approximately two-week intervals.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or 10 lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible.

15 Often, a panel of animals will have been immunized and the spleen of an animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

20 The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render them incapable of growing in certain selective 25 media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, 30 NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210;

and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed
5 P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

10 Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described
15 by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods also is appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about
20 1×10^{-6} to 1×10^{-8} . However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and
25 preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with
30 hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, e.g., hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate 5 this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific 10 hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the 15 like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in 20 two basic ways. First, a sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion (e.g., a syngeneic mouse). Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. The injected animal develops tumors 25 secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. Second, the individual cell lines could be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations.

30

MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or

affinity chromatography. Fragments of the monoclonal antibodies of the invention can be obtained from the monoclonal antibodies so produced by methods which include digestion with enzymes, such as pepsin or papain, and/or by cleavage of disulfide bonds by chemical reduction. Alternatively, monoclonal antibody fragments 5 encompassed by the present invention can be synthesized using an automated peptide synthesizer.

It also is contemplated that a molecular cloning approach may be used to generate monoclonals. For this, combinatorial immunoglobulin phagemid libraries are 10 prepared from RNA isolated from the spleen of the immunized animal, and phagemids expressing appropriate antibodies are selected by panning using cells expressing the antigen and control cells. The advantages of this approach over conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H 15 and L chain combination which further increases the chance of finding appropriate antibodies.

Humanized monoclonal antibodies are antibodies of animal origin that have been modified using genetic engineering techniques to replace constant region and/or 20 variable region framework sequences with human sequences, while retaining the original antigen specificity. Such antibodies are commonly derived from rodent antibodies with specificity against human antigens. Such antibodies are generally useful for *in vivo* therapeutic applications. This strategy reduces the host response to the foreign antibody and allows selection of the human effector functions.

25

The techniques for producing humanized immunoglobulins are well known to those of skill in the art. For example US Patent No. 5,693,762 discloses methods for producing, and compositions of, humanized immunoglobulins having one or more complementarity determining regions (CDR's). When combined into an intact 30 antibody, the humanized immunoglobulins are substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin to the antigen, such as a protein or other compound containing an epitope.

Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer, or by expression of full-length gene or of gene fragments in *E. coli*.

5

B. IGFBP-2 ANTIGENIC SEQUENCES

As another way of effecting modulation of IGFBP-2 in a subject, peptides corresponding to one or more antigenic determinants of the IGFBP-2 polypeptides of the present invention also can be prepared so that an immune response against IGFBP-10 2 is raised. Thus, it is contemplated that vaccination with an IGFBP-2 peptide or polypeptide may generate an autoimmune response in an immunized animal such that autoantibodies that specifically recognize the animal's endogenous IGFBP-2 protein. This vaccination technology is shown in U.S. Patent Nos. 6,027,727; 5,785,970, and 5,609,870, which are hereby incorporated by reference.

15

Such peptides should generally be at least five or six amino acid residues in length and will preferably be about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or about 30 amino acid residues in length, and may contain up to about 35-50 residues. For example, these peptides may comprise IGFBP-2 amino acid sequence, such as 20 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, and 50 or more contiguous amino acids from SEQ ID NO:2. Synthetic peptides will generally be about 35 residues long, which is the approximate upper length limit of automated peptide synthesis machines, such as those available from Applied Biosystems (Foster City, CA). Longer peptides also may be prepared, e.g., by 25 recombinant means.

U.S. Patent 4,554,101, (Hopp) incorporated herein by reference, teaches the identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed in Hopp, one of skill in the art 30 would be able to identify epitopes from within an amino acid sequence such as the IGFBP-2 sequence disclosed herein in SEQ ID NO: 2. A peptide with the sequence

LFYNEQQEARGVHTQRMQ (SEQ ID NO:3) has been used to generate antibodies against human IGFBP-2.

Numerous scientific publications have also been devoted to the prediction of
5 secondary structure, and to the identification of epitopes, from analyses of amino acid
sequences (Chou & Fasman, 1974a,b; 1978a,b, 1979). Any of these may be used, if
desired, to supplement the teachings of Hopp in U.S. Patent 4,554,101.

Moreover, computer programs are currently available to assist with predicting
10 antigenic portions and epitopic core regions of proteins. Examples include those
programs based upon the Jameson-Wolf analysis (Jameson & Wolf, 1988; Wolf *et al.*,
1988), the program PepPlot® (Brutlag *et al.*, 1990; Weinberger *et al.*, 1985), and
other new programs for protein tertiary structure prediction (Fetrow & Bryant, 1993).
15 Another commercially available software program capable of carrying out such
analyses is MacVector (IBI, New Haven, CT).

In further embodiments, major antigenic determinants of a IGFBP-2
polypeptide may be identified by an empirical approach in which portions of the gene
encoding the IGFBP-2 polypeptide are expressed in a recombinant host, and the
20 resulting proteins tested for their ability to elicit an immune response. For example,
PCR™ can be used to prepare a range of peptides lacking successively longer
fragments of the C-terminus of the protein. The immunoactivity of each of these
peptides is determined to identify those fragments or domains of the polypeptide that
are immunodominant. Further studies in which only a small number of amino acids are
25 removed at each iteration then allows the location of the antigenic determinants of the
polypeptide to be more precisely determined.

Another method for determining the major antigenic determinants of a
polypeptide is the SPOTs™ system (Genosys Biotechnologies, Inc., The Woodlands,
30 TX). In this method, overlapping peptides are synthesized on a cellulose membrane,
which following synthesis and deprotection, is screened using a polyclonal or

monoclonal antibody. The antigenic determinants of the peptides which are initially identified can be further localized by performing subsequent syntheses of smaller peptides with larger overlaps, and by eventually replacing individual amino acids at each position along the immunoreactive peptide.

5

Once one or more such analyses are completed, polypeptides are prepared that contain at least the essential features of one or more antigenic determinants. The peptides are then employed in the generation of antisera against the polypeptide. Minigenes or gene fusions encoding these determinants also can be constructed and 10 inserted into expression vectors by standard methods, for example, using PCR™ cloning methodology.

The use of such small peptides for antibody generation or vaccination typically requires conjugation of the peptide to an immunogenic carrier protein, such as hepatitis 15 B surface antigen, keyhole limpet hemocyanin or bovine serum albumin, or other adjuvants discussed above (adjuvenated peptide). Alum is an adjuvant that has proven sufficiently non-toxic for use in humans. Methods for performing this conjugation are well known in the art. Other immunopotentiating compounds are also contemplated 20 for use with the compositions of the invention such as polysaccharides, including chitosan, which is described in U.S. Patent No. 5,980,912, hereby incorporated by reference. Multiple (more than one) IGFBP-2 epitopes may be crosslinked to one another (e.g., polymerized). Alternatively, a nucleic acid sequence encoding an 25 IGFBP-2 peptide or polypeptide may be combined with a nucleic acid sequence that heightens the immune response. Such fusion proteins may comprise part or all of a foreign (non-self) protein such as bacterial sequences, for example.

Antibody titers effective to achieve a response against endogenous IGFBP-2 will vary with the species of the vaccinated animal, as well as with the sequence of the administered peptide. However, effective titers may be readily determined, for 30 example, by testing a panel of animals with varying doses of the specific antigen and measuring the induced titers of autoantibodies (or anti-self antibodies) by known

techniques, such as ELISA assays, and then correlating the titers with IGFBP-2-related cancer characteristics, *e.g.*, tumor growth or size.

One of ordinary skill would know various assays to determine whether an immune response against IGFBP-2 was generated. The phrase "immune response" includes both cellular and humoral immune responses. Various B lymphocyte and T lymphocyte assays are well known, such as ELISAs, cytotoxic T lymphocyte (CTL) assays, such as chromium release assays, proliferation assays using peripheral blood lymphocytes (PBL), tetramer assays, and cytokine production assays. See Benjamini *et al.*, 1991, hereby incorporated by reference.

C. LINKERS/COUPLING AGENTS

If desired, dimers or multimers of IGFBP-2 peptides, or an IGFBP-2 peptide and a non-self, non-IGFBP-2 peptide or adjuvant may be joined *via* a biologically-releasable bond, such as a selectively-cleavable linker or amino acid sequence. For example, peptide linkers that include a cleavage site for an enzyme preferentially located or active within a tumor environment are contemplated. Exemplary forms of such peptide linkers are those that are cleaved by urokinase, plasmin, thrombin, Factor IXa, Factor Xa, or a metallaproteinase, such as collagenase, gelatinase, or stromelysin. Alternatively, the linkers and coupling agents described herein may be employed to join an anti-IGFBP-2 antibody with a therapeutic or preventative agent, such as a toxin.

Amino acids such as selectively-cleavable linkers, synthetic linkers, or other amino acid sequences may be used to separate an IGFBP-2 peptide from another peptide or adjuvant, or anti-IGFBP-2 antibodies from a therapeutic compound.

Additionally, while numerous types of disulfide-bond containing linkers are known that can successfully be employed to conjugate the toxin moiety with the targeting agent, certain linkers will generally be preferred over other linkers, based on differing pharmacologic characteristics and capabilities. For example, linkers that contain a disulfide bond that is sterically "hindered" are to be preferred, due to their greater stability *in vivo*, thus preventing release of the toxin moiety prior to binding at

the site of action. Furthermore, while certain advantages in accordance with the invention will be realized through the use of any of a number of toxin moieties, the inventors have found that the use of ricin A chain, and even more preferably deglycosylated A chain, will provide particular benefits.

5

i) Biochemical cross-linkers

It can be considered as a general guideline that any biochemical cross-linker that is appropriate for use in an immunotoxin will also be of use in the present context, and additional linkers may also be considered.

10

Cross-linking reagents are used to form molecular bridges that tie together functional groups of two different molecules, e.g., a stabilizing and coagulating agent. To link two different proteins in a step-wise manner, hetero-bifunctional cross-linkers can be used that eliminate unwanted homopolymer formation.

TABLE 1
HETERO-BIFUNCTIONAL CROSS-LINKERS

linker	Reactive Toward	Advantages and Applications	Spacer Arm Length\after cross-linking
SMPT	Primary amines Sulphydryls	· Greater stability	11.2 Å
SPDP	Primary amines Sulphydryls	· Thiolation · Cleavable cross-linking	6.8 Å
LC-SPDP	Primary amines Sulphydryls	· Extended spacer arm	15.6 Å
Sulfo-LC-SPDP	Primary amines Sulphydryls	· Extended spacer arm · Water-soluble	15.6 Å
SMCC	Primary amines Sulphydryls	· Stable maleimide reactive group · Enzyme-antibody conjugation · Hapten-carrier protein conjugation	11.6 Å
Sulfo-SMCC	Primary amines Sulphydryls	· Stable maleimide reactive group · Water-soluble · Enzyme-antibody conjugation	11.6 Å
MBS	Primary amines Sulphydryls	· Enzyme-antibody conjugation · Hapten-carrier protein conjugation	9.9 Å
Sulfo-MBS	Primary amines Sulphydryls	· Water-soluble	9.9 Å
SIAB	Primary amines Sulphydryls	· Enzyme-antibody conjugation	10.6 Å
Sulfo-SIAB	Primary amines Sulphydryls	· Water-soluble	10.6 Å
SMPB	Primary amines Sulphydryls	· Extended spacer arm · Enzyme-antibody conjugation	14.5 Å
Sulfo-SMPB	Primary amines Sulphydryls	· Extended spacer arm · Water-soluble	14.5 Å
EDC/Sulfo-NHS	Primary amines Carboxyl groups	· Hapten-Carrier conjugation	0
ABH	Carbohydrates Nonselective	· Reacts with sugar groups	11.9 Å

An exemplary hetero-bifunctional cross-linker contains two reactive groups: one reacting with primary amine group (e.g., N-hydroxy succinimide) and the other reacting with a thiol group (e.g., pyridyl disulfide, maleimides, halogens, etc.). Through the primary amine reactive group, the cross-linker may react with the lysine residue(s) of one protein (e.g., the selected antibody or fragment) and through the thiol reactive group, the cross-linker, already tied up to the first protein, reacts with the cysteine residue (free sulfhydryl group) of the other protein (e.g., the selective agent).

It can therefore be seen that a targeted peptide composition will generally have, or be derivatized to have, a functional group available for cross-linking purposes. This requirement is not considered to be limiting in that a wide variety of groups can be used in this manner. For example, primary or secondary amine groups, hydrazide or hydrazine groups, carboxyl alcohol, phosphate, or alkylating groups may be used for binding or cross-linking. For a general overview of linking technology, one may wish to refer to Ghose & Blair (1987).

The spacer arm between the two reactive groups of a cross-linkers may have various length and chemical compositions. A longer spacer arm allows a better flexibility of the conjugate components while some particular components in the bridge (e.g., benzene group) may lend extra stability to the reactive group or an increased resistance of the chemical link to the action of various aspects (e.g., disulfide bond resistant to reducing agents). The use of peptide spacers, such as L-Leu-L-Ala-L-Leu-L-Ala, is also contemplated.

It is preferred that a cross-linker having reasonable stability in blood will be employed. Numerous types of disulfide-bond containing linkers are known that can be successfully employed to conjugate targeting and therapeutic/preventative agents. Linkers that contain a disulfide bond that is sterically hindered may prove to give greater stability *in vivo*, preventing release of the targeting peptide prior to reaching the site of action. These linkers are thus one group of linking agents.

Another cross-linking reagents for use in immunotoxins is SMPT, which is a bifunctional cross-linker containing a disulfide bond that is "sterically hindered" by an adjacent benzene ring and methyl groups. It is believed that stearic hindrance of the disulfide bond serves a function of protecting the bond from attack by thiolate anions 5 such as glutathione which can be present in tissues and blood, and thereby help in preventing decoupling of the conjugate prior to the delivery of the attached agent to the tumor site. It is contemplated that the SMPT agent may also be used in connection with the bispecific coagulating ligands of this invention.

10 The SMPT cross-linking reagent, as with many other known cross-linking reagents, lends the ability to cross-link functional groups such as the SH of cysteine or primary amines (*e.g.*, the epsilon amino group of lysine). Another possible type of cross-linker includes the hetero-bifunctional photoreactive phenylazides containing a cleavable disulfide bond such as sulfosuccinimidyl-2-(*p*-azido salicylamido) ethyl-1,3'-dithiopropionate. The N-hydroxy-succinimidyl group reacts with primary amino 15 groups and the phenylazide (upon photolysis) reacts non-selectively with any amino acid residue.

20 In addition to hindered cross-linkers, non-hindered linkers also can be employed in accordance herewith. Other useful cross-linkers, not considered to contain or generate a protected disulfide, include SATA, SPDP and 2-iminothiolane (Wawrzynczak & Thorpe, 1988). The use of such cross-linkers is well understood in the art.

25 Once conjugated, the peptide generally will be purified to separate the conjugate from unconjugated targeting agents or coagulants and from other contaminants. A large a number of purification techniques are available for use in providing conjugates of a sufficient degree of purity to render them clinically useful. Purification methods based upon size separation, such as gel filtration, gel permeation 30 or high performance liquid chromatography, will generally be of most use. Other chromatographic techniques, such as Blue-Sepharose separation, may also be used.

In addition to chemical conjugation, an IGFBP-2 polypeptide, peptide, or antibody may be modified at the protein level. Included within the scope of the invention are IgA protein fragments or other derivatives or analogs that are differentially modified during or after translation, for example by glycosylation, 5 acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, and proteolytic cleavage. Any number of chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, farnesylation, oxidation, reduction; metabolic synthesis in the 10 presence of tunicamycin.

3. IGFBP-2 NUCLEIC ACIDS

A. GENES AND DNA SEGMENTS

Important aspects of the present invention concern isolated DNA segments and 15 recombinant vectors expressing IGFBP-2 antisense or sense, and the creation and use of recombinant host cells through the application of DNA technology, including such sequences encoding one or more IGFBP-2 polypeptides, or functional equivalents thereof.

20 The present invention concerns DNA segments, isolatable from mammalian and human cells, that are free from total genomic DNA and are capable of expressing IGFBP-2 polypeptide in a recombinant host cell when incorporated into the recombinant host cell.

25 As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding IGFBP-2 refers to a DNA segment that contains coding sequences of IGFBP-2, yet is isolated away from, or purified free from, total mammalian or 30 human genomic DNA. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified *IGFBP-2* gene refers to a DNA segment including any of the *IGFBP-2* gene coding sequences and, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, cDNA sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides or fusion proteins.

10

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case any *IGFBP-2* gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode an *IGFBP-2* polypeptide that includes within its amino acid sequence a contiguous amino acid sequence of human *IGFBP-2* polypeptide, or functional equivalents thereof.

Naturally, where the DNA segment or vector encodes an *IGFBP-2* polypeptide, or is intended for use in expressing the *IGFBP-2* polypeptide, the most preferred sequences are those that are essentially as set forth in the contiguous sequence of SEQ ID NO:1.

Sequence of an *IGFBP-2* polypeptide will substantially correspond to a contiguous portion of that shown in SEQ ID NO:2, and have relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino

acids shown in SEQ ID NO:2. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein.

Accordingly, sequences that have between about 70% and about 80%; or more 5 preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:2 will be sequences that are "essentially as set forth in SEQ ID NO:2."

10 In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that include within their sequence a contiguous nucleic acid sequence from that shown in SEQ ID NO:1. This definition is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a contiguous portion of that shown in SEQ ID NO:1 and has relatively few codons 15 that are not identical, or functionally equivalent, to the codons of SEQ ID NO:1. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids. See Table 2 below, which lists the codons preferred for use in humans, with the codons listed in decreasing order of 20 preference from left to right in the table (Wada *et al.*, 1990). Codon preferences for other organisms also are well known to those of skill in the art (Wada *et al.*, 1990, included herein in its entirety by reference).

Table 2
Preferred Human DNA Codons

<u>Amino Acids</u>		<u>Codons</u>					
Alanine	Ala	A	GCC	GCT	GC	GCG	
					A		
Cysteine	Cys	C	TGC	TGT			
Aspartic acid	Asp	D	GAC	GAT			
Glutamic acid	Glu	E	GAG	GAA			
Phenylalanine	Phe	F	TTC	TTT			
Glycine	Gly	G	GGC	GGG	GG	GGT	
					A		
Histidine	His	H	CAC	CAT			
Isoleucine	Ile	I	ATC	ATT	AT		
					A		
Lysine	Lys	K	AAG	AAA			
Leucine	Leu	L	CTG	CTC	TTG	CTT	CTA TTA
Methionine	Met	M	ATG				
Asparagine	Asn	N	AAC	AAT			
Proline	Pro	P	CCC	CCT	CC	CCG	
					A		
Glutamine	Gln	Q	CAG	CAA			
Arginine	Arg	R	CGC	AGG	CG	AGA	CGA CGT
					G		
Serine	Ser	S	AGC	TCC	TCT	AGT	TCA TCG
Threonine	Thr	T	ACC	ACA	AC	ACG	
					T		
Valine	Val	V	GTG	GTC	GTT	GTA	
Tryptophan	Trp	W	TGG				
Tyrosine	Tyr	Y	TAC	TAT			

It also will be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the

maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns,
5 which are known to occur within genes.

Excepting intronic or flanking regions, and allowing for the degeneracy of the genetic code, sequences that have between about 70% and about 79%; or more preferably, between about 80% and about 89%; or even more preferably, between
10 about 90% and about 99% of nucleotides that are identical to the nucleotides shown in the sequences of SEQ ID NO:1 will be sequences that are "essentially as set forth in SEQ ID NO:1". Sequences that are essentially the same as those set forth in SEQ ID NO:1 also may be functionally defined as sequences that are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NO:1 under relatively
15 stringent conditions. Suitable relatively stringent hybridization conditions will be well known to those of skill in the art.

Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:1. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid
25 segment of SEQ ID NO:1 under relatively stringent conditions.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning
30 sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of

preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared that include a short contiguous stretch identical to or complementary to the sequence shown in SEQ ID NO:1, such as about 14 nucleotides, and that are up to about 10,000 or about 5,000 base pairs in length, with segments of 5 about 3,000 being preferred in certain cases. DNA segments with total lengths of about 1,000, about 500, about 200, about 100 and about 50 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

It will be readily understood that "intermediate lengths", in these contexts, 10 means any length between the quoted ranges, such as 14, 15, 16, 17, 18, 19, 20, etc.; 21, 22, 23, etc.; 30, 31, 32, etc.; 50, 51, 52, 53, etc.; 100, 101, 102, 103, etc.; 150, 151, 152, 153, etc.; including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000 ranges, up to and including sequences of about 12,001, 12,002, 13,001, 13,002 and the like.

15

The various probes and primers designed around the nucleotide sequences of the present invention may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, etc., an algorithm defining all primers can be proposed:

20

n to n + y

where n is an integer from 1 to the last number of the sequence and y is the length of the primer minus one, where n + y does not exceed the last number of the sequence. Thus, for a 10-mer, the probes correspond to bases 1 to 10, 2 to 11, 3 to 12 ... and so on. For a 15-mer, the probes correspond to bases 1 to 15, 2 to 16, 3 to 17 ... and so on. For a 25 20-mer, the probes correspond to bases 1 to 20, 2 to 21, 3 to 22 ... and so on.

It also will be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:1 and SEQ ID NO:2. Recombinant vectors and isolated DNA segments may therefore variously include the 30 IGFBP-2 coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that

nevertheless include IGFBP-2 -coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

The DNA segments of the present invention encompass biologically functional equivalent IGFBP-2 proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the antigenicity of the protein.

If desired, one also may prepare fusion proteins and peptides, e.g., where the IGFBP-2 coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (e.g., proteins that may be purified by affinity chromatography and enzyme label coding regions, respectively).

20

Encompassed by certain embodiments of the present invention are DNA segments encoding relatively small peptides, such as, for example, peptides of from about 15 to about 50 amino acids in length, and more preferably, of from about 15 to about 30 amino acids in length; and also larger polypeptides up to and including proteins corresponding to the full-length sequences set forth in SEQ ID NO:2, or to specific fragments of SEQ ID NO:1 that correspond to differences as compared to the published sequence for IGFBP-2.

B. AMPLIFICATION AND PCR™

Nucleic acids used as a template for amplification are isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell

RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

5 Pairs of primers that selectively hybridize to nucleic acids corresponding to *IGFBP-2* are contacted with the isolated nucleic acid under conditions that permit selective hybridization. The term "primer", as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to
10 twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

Once hybridized, the nucleic acid:primer complex is contacted with one or
15 more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the
20 detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals.

25 A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCRTM) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and each incorporated herein by reference in entirety.

Briefly, in PCR™, two or more primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, e.g., *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

10

A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641, filed December 21, 1990, incorporated herein by reference. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPA No. 320 308, incorporated herein by reference in its entirety. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, incorporated herein by reference, also may be used as still another amplification method in the present invention.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 30'-[alpha-thio]-triphosphates in one strand of a restriction site also may be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair 5 Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences also can be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' 10 sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

15

Still another amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, 20 template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled 25 probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR Gingeras *et al.*, PCT Application WO 88/10315, incorporated 30 herein by reference.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

5

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This 10 scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, 1990 incorporated by reference).

Methods based on ligation of two (or more) oligonucleotides in the presence of 15 nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, also may be used in the amplification step of the present invention.

Following any amplification, it may be desirable to separate the amplification 20 product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See Sambrook *et al.*, 1989.

25 Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

30

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with

ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

5

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding 10 partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those 15 of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose or nylon, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of 20 hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U.S. Patent 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the 25 automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

4. RECOMBINANT VECTORS, HOST CELLS AND EXPRESSION

Recombinant vectors form important further aspects of the present invention. The term "expression vector or construct" means any type of genetic construct 30

containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed and subsequently translated.

Particularly useful vectors are contemplated to be those vectors in which the
5 coding portion of the DNA segment, whether encoding a full length protein or smaller peptide, is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned", "under control", "operably linked to" or "under
10 transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The promoter may be in the form of the promoter that is naturally associated
15 with any of the *IGFBP-2* genes, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR technology, in connection with the compositions disclosed herein (PCR technology is disclosed in U.S. Patent 4,683,202 and U.S. Patent 4,683,195, each incorporated herein by reference).

20

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an *IGFBP-2* gene
25 in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell.

Naturally, it will be important to employ a promoter that effectively directs the
30 expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see

Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. In a preferred 5 embodiment, the promoter preferentially expresses or is specific for a tissue or cell type, such as a cell type that expresses IGFBP-2. It is contemplated that such cell types may be from, but not limited to, prostate and breast tissues, and may be altered or cancerous.

10 At least one module in a promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

15 Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements 20 frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

25 The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is 30 capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known 5 in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 3 and 4 below list several enhancers, promoters and inducible elements which may be employed, in the context of the present invention, to regulate the expression of any of the *IGFBP-2* constructs. This list is not intended to be exhaustive of all the possible elements 10 involved in the promotion of transgene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of 15 DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which serves as a binding region for one or more transcriptional proteins.

20

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a 25 particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic 30 Promoter Data Base EPDB) could also be used to drive expression of a transgene. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain

bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

Table 3
Promoter and Enhancer Elements

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl and Baltimore, 1985; Atchison and Perry, 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1984; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> ; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987; Winoto and Baltimore, 1989; Redondo <i>et al.</i> ; 1990
HLA DQ α and DQ β	Sullivan and Peterlin, 1987
β -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-DRa	Sherman <i>et al.</i> , 1989
β -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> ; 1989
Muscle Creatine Kinase	Jaynes <i>et al.</i> , 1988; Horlick and Benfield, 1989; Johnson <i>et al.</i> , 1989
Prealbumin (Transthyretin)	Costa <i>et al.</i> , 1988
Elastase I	Ornitz <i>et al.</i> , 1987
Metallothionein	Karin <i>et al.</i> , 1987; Culotta and Hamer, 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987

TABLE 3 - CONTINUED

Albumin Gene	Pinkert <i>et al.</i> , 1987; Tronche <i>et al.</i> , 1989, 1990
α -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β -Globin	Trudel and Constantini, 1987
e-fos	Cohen <i>et al.</i> , 1978
c-HA-ras	Triesman, 1985; Deschamps <i>et al.</i> , 1985
Insulin	Edlund <i>et al.</i> , 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh <i>et al.</i> , 1990
α_1 -Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy	Klamut <i>et al.</i> , 1990

TABLE 3 - CONTINUED

SV40	Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987; Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndall <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; de Villiers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a, b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander and Haseltine, 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Choi <i>et al.</i> , 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky and Botchan, 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987; Stephens and Hentschel, 1987; Gius <i>et al.</i> , 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988

TABLE 3 - CONTINUED

Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber and Cullan, 1988; Jakobovits <i>et al.</i> , 1988; Feng and Holland, 1988; Takebe <i>et al.</i> , 1988; Rosen <i>et al.</i> , 1988; Berkhouit <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp and Marciniak, 1989; Braddock <i>et al.</i> , 1989
Cytomegalovirus	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

Table 4
Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA)	Palmiter <i>et al.</i> , 1982;
	Heavy metals	Haslinger and Karin, 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987; Karin <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987b; McNeall <i>et al.</i> , 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang <i>et al.</i> , 1981; Lee <i>et al.</i> , 1981; Majors and Varmus, 1983; Chandler <i>et al.</i> , 1983; Ponta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 <u>E2</u>	Ela	Imperiale and Nevins, 1984

TABLE 4 - CONTINUED

Collagenase	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
SV40	Phorbol Ester (TPA)	Angel <i>et al.</i> , 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
α -2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2kb	Interferon	Blanar <i>et al.</i> , 1989
HSP70	Ela, SV40 Large T Antigen	Taylor <i>et al.</i> , 1989; Taylor and Kingston, 1990a, b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

Turning to the expression of IGFBP-2 polypeptides, once a suitable clone or clones have been obtained, whether they be cDNA based or genomic, one may proceed to prepare an expression system. The engineering of DNA segment(s) for expression in a prokaryotic or eukaryotic system may be performed by techniques generally known to those of skill in recombinant expression. It is believed that virtually any expression system may be employed in the expression of the proteins of the present invention.

Both cDNA and genomic sequences are suitable for eukaryotic expression, as the host cell will generally process the genomic transcripts to yield functional mRNA for translation into protein. Generally speaking, it may be more convenient to employ as the recombinant gene a cDNA version of the gene. It is believed that the use of a cDNA version will provide advantages in that the size of the gene will generally be

much smaller and more readily employed to transfect the targeted cell than will a genomic gene, which will typically be up to an order of magnitude larger than the cDNA gene. However, the inventors do not exclude the possibility of employing a genomic version of a particular gene where desired.

5

In expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation 10 signal and the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

15

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that 20 the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. It is known that the primary sequence surrounding the ATG initiation codon $\text{GCC}(\text{A})\text{CCATGG}$ is the optimal context for initiation of translation in higher eukaryotes (Kozak 1991). Thus 25 mutagenesis of the sequence surrounding the ATG codon may be mutated, as described in detail herein, is contemplated by the inventors as a mechanism to improve the efficiency of translation of the *IGFBP-2* constructs of the present invention.

30

As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a cell into which an exogenous DNA segment or gene, such as a cDNA or gene encoding an *IGFBP-2* polypeptide has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a

recombinantly introduced exogenous DNA segment or gene. Engineered cells are thus cells having a gene or genes introduced through the hand of man. Recombinant cells include those having an introduced cDNA or genomic gene, and also include genes positioned adjacent to a promoter not naturally associated with the particular 5 introduced gene.

To express a recombinant IGFBP-2 protein, whether mutant or wild-type, in accordance with the present invention one would prepare an expression vector that comprises a IGFBP-2-encoding nucleic acid under the control of one or more 10 promoters. To bring a coding sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded recombinant protein. This is the meaning of 15 "recombinant expression" in this context.

5. ANTISENSE AND RIBOZYMES

A. ANTISENSE

20 Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and 25 adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

30 Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target

polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

5

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs may include regions complementary to intron/exon splice junctions. Thus, antisense constructs with 10 complementarity to regions within 50-200 bases of an intron-exon splice junction may be used. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of 15 exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide 20 sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have 25 no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (e.g., ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

30

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is

desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

5

B. RIBOZYMES

The use of IGFBP-2-specific ribozymes is claimed in the present application. The following information is provided in order to compliment the earlier section and to assist those of skill in the art in this endeavor.

10

Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlack *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with 15 a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

20

Ribozyme catalysis has primarily been observed as part of sequence specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U.S. Patent 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and 25 approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990; Sioud *et al.*, 1992). Recently, it was reported that ribozymes elicited genetic changes in some cell lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. 30 Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme. In light of the information included herein and the knowledge of one of ordinary skill in the art, the preparation

and use of additional ribozymes that are specifically targeted to a given gene will now be straightforward.

Several different ribozyme motifs have been described with RNA cleavage activity (reviewed in Symons, 1992). Examples that would be expected to function equivalently for the down regulation of IGFBP-2 include sequences from the Group I self splicing introns including tobacco ringspot virus (Prody *et al.*, 1986), avocado sunblotch viroid (Palukaitis *et al.*, 1979 and Symons, 1981), and Lucerne transient streak virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead ribozymes based on a predicted folded secondary structure.

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity (Yuan *et al.*, 1992, Yuan and Altman, 1994), hairpin ribozyme structures (Berzal-Herranz *et al.*, 1992; Chowrira *et al.*, 1993) and hepatitis delta virus based ribozymes (Perrotta and Been, 1992). The general design and optimization of ribozyme directed RNA cleavage activity has been discussed in detail (Haseloff and Gerlach, 1988, Symons, 1992, Chowrira, *et al.*, 1994, and Thompson, *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to a site by complimentary base pair interactions. Two stretches of homology are required for this targeting. These stretches of homologous sequences flank the catalytic ribozyme structure defined above. Each stretch of homologous sequence can vary in length from 7 to 15 nucleotides. The only requirement for defining the homologous sequences is that, on the target RNA, they are separated by a specific sequence which is the cleavage site. For hammerhead ribozymes, the cleavage site is a dinucleotide sequence on the target RNA, uracil (U) followed by either an adenine, cytosine or uracil (A,C or U; Perriman, *et al.*, 1992; Thompson, *et al.*, 1995). The frequency of this dinucleotide occurring in any given RNA is statistically 3 out of 16. Therefore, for a given target messenger RNA of 1000 bases, 187 dinucleotide cleavage sites are statistically possible. The message for IGFBP-2 targeted here are greater than 1400 bases long, with greater than 260 possible cleavage sites.

The large number of possible cleavage sites in the IGFBP-2 coupled with the growing number of sequences with demonstrated catalytic RNA cleavage activity indicates that a large number of ribozymes that have the potential to downregulate the 5 IGFBP-2 are available. Designing and testing ribozymes for efficient cleavage of a target RNA is a process well known to those skilled in the art. Examples of scientific methods for designing and testing ribozymes are described by Chowrira *et al.*, (1994) and Lieber and Strauss (1995), each incorporated by reference. The identification of operative and preferred sequences for use in IGFBP-2-targeted ribozymes is simply a 10 matter of preparing and testing a given sequence, and is a routinely practiced "screening" method known to those of skill in the art.

C. SINGLE-CHAIN ANTIBODIES

Another therapeutic embodiment of the present invention contemplates the use 15 of single-chain antibodies to block the activity of IGFBP-2 in cells, in particular cancer cells. Single-chain antibodies can be synthesized by a cell, targeted to particular cellular compartments, and used to interfere in a highly specific manner with cell growth and metabolism (Richardson and Marasco, 1995). Recently, single-chain antibodies were utilized for the phenotypic knockout of growth-factor receptors, the 20 functional inactivation of p21ras, and the inhibition of HIV-1 replication. Intracellular antibodies offer a simple and effective alternative to other forms of gene inactivation, as well as demonstrate a clear potential as reagents for cancer therapy and for the control of infectious diseases. Single-chain antigen-binding proteins also represent potentially unique molecules for targeted delivery of drugs, toxins, or radionuclides to 25 a tumor site, and show increased accessibility to tumor cells *in vivo* (Yokoda *et al.*, 1992). Single-chain antibodies that bind IGFBP-2 can be introduced into a cell to functionally inactivate the IGFBP-2.

Methods for the production of single-chain antibodies are well known to those 30 of skill in the art. The skilled artisan is referred to U.S. Patent 5,359,046, (incorporated herein by reference) for such methods. A single-chain antibody is

created by fusing together the variable domains of the heavy and light chains using a short peptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (scFvs) in which the C-terminus of 5 one variable domain is tethered to the N-terminus of the other *via* a 15 to 25 amino acid peptide or linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk *et al.*, 1990; Chaudhary *et al.*, 1990). These Fvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody.

10

It is also contemplated by the present invention that single-chain antibody therapy can be combined with chemotherapeutic or radiotherapeutic intervention. The discussion of combined therapy with traditional chemotherapy or radiotherapy employed herein is incorporated into this section by reference.

15

6. METHODS OF GENE TRANSFER

In order to mediate the effect transgene expression in a cell, it will be necessary to transfer the therapeutic expression constructs of the present invention into a cell. Such transfer may employ viral or non-viral methods of gene transfer. This section 20 provides a discussion of methods and compositions of gene transfer.

A. Viral Vector-Mediated Transfer

The IGFBP-2 genes are incorporated into an adenoviral infectious particle to mediate gene transfer to a cell. Additional expression constructs encoding other 25 therapeutic agents as described herein may also be transferred *via* viral transduction using infectious viral particles, for example, by transformation with an adenovirus vector of the present invention as described herein below. Alternatively, retroviral or bovine papilloma virus may be employed, both of which permit permanent transformation of a host cell with a gene(s) of interest. Thus, in one example, viral 30 infection of cells is used in order to deliver therapeutically significant genes to a cell. Typically, the virus simply will be exposed to the appropriate host cell under physiologic conditions, permitting uptake of the virus. Though adenovirus is

exemplified, the present methods may be advantageously employed with other viral vectors, as discussed below.

i) *Adenovirus.*

5 Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized DNA genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. The roughly 36 kB viral genome is bounded by 100-200 base pair (bp) inverted terminal repeats (ITR), in which are contained *cis*-acting elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions
10 of the genome that contain different transcription units are divided by the onset of viral DNA replication.

The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression, and host cell shut off (Renan, 1990). The products of the late genes (L1, L2, L3, L4 and L5), including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter
20 (MLP). The MLP (located at 16.8 map units) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5' tripartite leader (TL) sequence which makes them preferred mRNAs for translation.

In order for adenovirus to be optimized for gene therapy, it is necessary to
25 maximize the carrying capacity so that large segments of DNA can be included. It also is very desirable to reduce the toxicity and immunologic reaction associated with certain adenoviral products. The two goals are, to an extent, coterminous in that elimination of adenoviral genes serves both ends. By practice of the present invention, it is possible achieve both these goals while retaining the ability to manipulate the
30 therapeutic constructs with relative ease.

The large displacement of DNA is possible because the *cis* elements required for viral DNA replication all are localized in the inverted terminal repeats (ITR) (100-200 bp) at either end of the linear viral genome. Plasmids containing ITR's can replicate in the presence of a non-defective adenovirus (Hay *et al.*, 1984). Therefore, 5 inclusion of these elements in an adenoviral vector should permit replication.

In addition, the packaging signal for viral encapsidation is localized between 194-385 bp (0.5-1.1 map units) at the left end of the viral genome (Hearing *et al.*, 1987). This signal mimics the protein recognition site in bacteriophage λ DNA where 10 a specific sequence close to the left end, but outside the cohesive end sequence, mediates the binding to proteins that are required for insertion of the DNA into the head structure. E1 substitution vectors of Ad have demonstrated that a 450 bp (0-1.25 map units) fragment at the left end of the viral genome could direct packaging in 293 cells (Levrero *et al.*, 1991).

15

Previously, it has been shown that certain regions of the adenoviral genome can be incorporated into the genome of mammalian cells and the genes encoded thereby expressed. These cell lines are capable of supporting the replication of an adenoviral vector that is deficient in the adenoviral function encoded by the cell line. There also 20 have been reports of complementation of replication deficient adenoviral vectors by "helping" vectors, *e.g.*, wild-type virus or conditionally defective mutants.

Replication-deficient adenoviral vectors can be complemented, in *trans*, by helper virus. This observation alone does not permit isolation of the replication- 25 deficient vectors, however, since the presence of helper virus, needed to provide replicative functions, would contaminate any preparation. Thus, an additional element was needed that would add specificity to the replication and/or packaging of the replication-deficient vector. That element, as provided for in the present invention, derives from the packaging function of adenovirus.

30

It has been shown that a packaging signal for adenovirus exists in the left end of the conventional adenovirus map (Tibbetts, 1977). Later studies showed that a

mutant with a deletion in the E1A (194-358 bp) region of the genome grew poorly even in a cell line that complemented the early (E1A) function (Hearing and Shenk, 1983). When a compensating adenoviral DNA (0-353 bp) was recombined into the right end of the mutant, the virus was packaged normally. Further mutational analysis 5 identified a short, repeated, position-dependent element in the left end of the Ad5 genome. One copy of the repeat was found to be sufficient for efficient packaging if present at either end of the genome, but not when moved towards the interior of the Ad5 DNA molecule (Hearing *et al.*, 1987).

10 By using mutated versions of the packaging signal, it is possible to create helper viruses that are packaged with varying efficiencies. Typically, the mutations are point mutations or deletions. When helper viruses with low efficiency packaging are grown in helper cells, the virus is packaged, albeit at reduced rates compared to wild-type virus, thereby permitting propagation of the helper. When these helper viruses are 15 grown in cells along with virus that contains wild-type packaging signals, however, the wild-type packaging signals are recognized preferentially over the mutated versions. Given a limiting amount of packaging factor, the virus containing the wild-type signals are packaged selectively when compared to the helpers. If the preference is great enough, stocks approaching homogeneity should be achieved.

20

ii) Retrovirus.

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process 25 of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes - gag, pol and env - that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene, termed Ψ , functions as a signal for 30 packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and

enhancer sequences and also are required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a promoter is
5 inserted into the viral genome in the place of certain viral sequences to produce a virus
that is replication-defective. In order to produce virions, a packaging cell line
containing the gag, pol and env genes but without the LTR and Ψ components is
constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a human
cDNA, together with the retroviral LTR and Ψ sequences is introduced into this cell
10 line (by calcium phosphate precipitation for example), the Ψ sequence allows the RNA
transcript of the recombinant plasmid to be packaged into viral particles, which are
then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986;
Mann *et al.*, 1983). The media containing the recombinant retroviruses is collected,
optionally concentrated, and used for gene transfer. Retroviral vectors are able to
15 infect a broad variety of cell types. However, integration and stable expression of
many types of retroviruses require the division of host cells (Paskind *et al.*, 1975).

An approach designed to allow specific targeting of retrovirus vectors recently
was developed based on the chemical modification of a retrovirus by the chemical
20 addition of galactose residues to the viral envelope. This modification could permit the
specific infection of cells such as hepatocytes *via* asialoglycoprotein receptors, should
this be desired.

A different approach to targeting of recombinant retroviruses was designed in
25 which biotinylated antibodies against a retroviral envelope protein and against a
specific cell receptor were used. The antibodies were coupled *via* the biotin
components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major
histocompatibility complex class I and class II antigens, the infection of a variety of
human cells that bore those surface antigens was demonstrated with an ecotropic virus
30 *in vitro* (Roux *et al.*, 1989).

iii) Adeno-associated Virus.

AAV utilizes a linear, single-stranded DNA of about 4700 base pairs. Inverted terminal repeats flank the genome. Two genes are present within the genome, giving rise to a number of distinct gene products. The first, the *cap* gene, produces three 5 different virion proteins (VP), designated VP-1, VP-2 and VP-3. The second, the *rep* gene, encodes four non-structural proteins (NS). One or more of these *rep* gene products is responsible for transactivating AAV transcription.

10 The three promoters in AAV are designated by their location, in map units, in the genome. These are, from left to right, p5, p19 and p40. Transcription gives rise to six transcripts, two initiated at each of three promoters, with one of each pair being spliced. The splice site, derived from map units 42-46, is the same for each transcript. The four non-structural proteins apparently are derived from the longer of the transcripts, and three virion proteins all arise from the smallest transcript.

15

AAV is not associated with any pathologic state in humans. Interestingly, for efficient replication, AAV requires "helping" functions from viruses such as herpes simplex virus I and II, cytomegalovirus, pseudorabies virus and, of course, adenovirus. The best characterized of the helpers is adenovirus, and many "early" functions for this 20 virus have been shown to assist with AAV replication. Low level expression of AAV *rep* proteins is believed to hold AAV structural expression in check, and helper virus infection is thought to remove this block.

25 The terminal repeats of the AAV vector can be obtained by restriction endonuclease digestion of AAV or a plasmid such as p201, which contains a modified AAV genome (Samulski *et al.* 1987), or by other methods known to the skilled artisan, including but not limited to chemical or enzymatic synthesis of the terminal repeats based upon the published sequence of AAV. The ordinarily skilled artisan can determine, by well-known methods such as deletion analysis, the minimum sequence or 30 part of the AAV ITRs which is required to allow function, *i.e.*, stable and site-specific integration. The ordinarily skilled artisan also can determine which minor

modifications of the sequence can be tolerated while maintaining the ability of the terminal repeats to direct stable, site-specific integration.

AAV-based vectors have proven to be safe and effective vehicles for gene delivery *in vitro*, and these vectors are being developed and tested in pre-clinical and clinical stages for a wide range of applications in potential gene therapy, both *ex vivo* and *in vivo* (Flotte and Carter, 1995; Chatterjee *et al.*, 1995; Ferrari *et al.*, 1996; Fisher *et al.*, 1996; Flotte *et al.*, 1993; Goodman *et al.*, 1994; Kaplitt *et al.*, 1994; 1996, Kessler *et al.*, 1996; Koeberl *et al.*, 1997; Mizukami *et al.*, 1996; Xiao *et al.*, 10 1996).

AAV-mediated efficient gene transfer and expression in the lung has led to clinical trials for the treatment of cystic fibrosis (Flotte and Carter, 1995; Flotte *et al.*, 1993). Similarly, the prospects for treatment of muscular dystrophy by AAV-mediated gene delivery of the dystrophin gene to skeletal muscle, of Parkinson's disease by tyrosine hydroxylase gene delivery to the brain, of hemophilia B by Factor IX gene delivery to the liver, and potentially of myocardial infarction by vascular endothelial growth factor gene to the heart, appear promising since AAV-mediated transgene expression in these organs has recently been shown to be highly efficient (Fisher *et al.*, 15 1996; Flotte *et al.*, 1993; Kaplitt *et al.*, 1994; 1996; Koeberl *et al.*, 1997; McCown *et al.*, 1996; Ping *et al.*, 1996; Xiao *et al.*, 1996).

iv) Other Viral Vectors.

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) canary pox virus, Ebstein Barr Virus, and herpesviruses may be employed. These viruses offer several features for use in gene transfer into various mammalian cells.

B. Non-viral Transfer

DNA constructs of the present invention are generally delivered to a cell, in certain situations, the nucleic acid to be transferred is non-infectious, and can be transferred using non-viral methods.

5

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979), cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988).

15

Once the construct has been delivered into the cell the nucleic acid encoding the therapeutic gene may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the therapeutic gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In a particular embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution.

The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). The addition of DNA to cationic liposomes causes a topological transition from liposomes to optically birefringent liquid-crystalline 5 condensed globules (Radler *et al.*, 1997). These DNA-lipid complexes are potential non-viral vectors for use in gene therapy.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Using the β -lactamase gene, Wong *et al.* (1980) 10 demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa, and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection. Also included are various commercial approaches involving "lipofection" technology.

15

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in 20 conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention.

25

Other vector delivery systems which can be employed to deliver a nucleic acid encoding a therapeutic gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution 30 of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and 5 transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

10 In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a therapeutic gene also may be specifically delivered into a cell 15 type such as prostate, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, the human prostate-specific antigen (Watt *et al.*, 1986) may be used as the receptor for mediated delivery of a nucleic acid in prostate tissue.

20 In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is applicable particularly for transfer *in vitro*, however, it may be applied for *in vivo* use as well. Dubensky *et al.* (1984) successfully 25 injected polyomavirus DNA in the form of CaPO₄ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of CaPO₄ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a CAM may also be transferred in a similar manner *in* 30 *vivo* and express CAM.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads

10 7. **IMMUNOTHERAPY**

The results presented herein have significant relevance to immunotherapy of human diseases and disorders, including cancer. In using the immunotherapeutic compositions derived from the IGFBP-2 polypeptides and peptides of the present invention in treatment methods, other standard treatments also may be employed, such 15 as radiotherapy or chemotherapy. However, it is preferred that the immunotherapy be used alone initially as its effectiveness can be readily assessed. Immunotherapies of cancer can broadly be classified as adoptive, passive and active, as described in the following sections.

20 It is contemplated that a wide variety of cancers may be treated using therapies described herein. Such cancers include but are not limited to cancers of the brain (*e.g.*, glioblastoma, astrocytoma, oligodendrogloma, ependymomas), neurofibrosarcoma, meningia, lung, liver, spleen, kidney, lymph node, pancreas, small intestine, blood cells, colon or colorectal region, stomach, thyroid, breast, endometrium, prostate, testes, 25 ovary, skin, head and neck, esophagus, bone marrow, blood or other tissue.

A. PASSIVE IMMUNOTHERAPY

A number of different approaches for passive immunotherapy of cancer exist. They may be broadly categorized into the following: injection of antibodies alone; 30 injection of antibodies coupled to toxins or chemotherapeutic agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotypic antibodies; and finally, purging of tumor cells in bone marrow.

Preferably, human monoclonal antibodies are employed in passive immunotherapy, as they produce few or no side effects in the patient. However, their application is somewhat limited by their scarcity and have so far only been
5 administered intralesionally. Human monoclonal antibodies to ganglioside antigens have been administered intralesionally to patients suffering from cutaneous recurrent melanoma (Irie & Morton, 1986). Regression was observed in six out of ten patients, following, daily or weekly, intralesional injections. In another study, moderate success was achieved from intralesional injections of two human monoclonal antibodies (Irie *et*
10 *al.*, 1989).

It may be favorable to administer more than one monoclonal antibody directed against two different antigens or even antibodies with multiple antigen specificity. Treatment protocols also may include administration of lymphokines or other immune
15 enhancers as described by Bajorin *et al.* (1988). The development of human monoclonal antibodies is described in further detail elsewhere in the specification.

B. ACTIVE IMMUNOTHERAPY

In active immunotherapy, an antigenic peptide, polypeptide or protein, or an
20 autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath & Morton, 1991; Morton & Ravindranath, 1996; Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993). In melanoma immunotherapy, those patients who elicit high IgM response often survive better than those who elicit no or low IgM antibodies (Morton *et al.*, 1992).
25 IgM antibodies are often transient antibodies and the exception to the rule appears to be anti-ganglioside or ant(carbohydrate) antibodies.

C. ADOPTIVE IMMUNOTHERAPY

In adoptive immunotherapy, the patient's circulating lymphocytes, or tumor
30 infiltrated lymphocytes, are isolated *in vitro*, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered (Rosenberg *et al.*, 1988; 1989). To achieve this, one would administer to an animal, or human patient, an

immunologically effective amount of activated lymphocytes in combination with an adjuvant-incorporated antigenic peptide composition as described herein. The activated lymphocytes will most preferably be the patient's own cells that were earlier isolated from a blood or tumor sample and activated (or "expanded") *in vitro*. This form of immunotherapy has produced several cases of regression of melanoma and renal carcinoma, but the percentage of responders were few compared to those who did not respond.

8. METHODS OF TREATING CANCER

In a particular aspect, the present invention provides methods for the treatment of various malignancies involving IGFBP-2 directed therapy, which includes compounds or compositions that affect IGFBP-2. Treatment methods will involve treating an individual with an effective amount of a viral particle, as described above, containing a therapeutic gene of interest. Other treatment methods involve treating an individual with an effective amount of an antibody. Alternatively, treatment may involve administering an IGFBP-2 peptide or polypeptide that elicits an immune response, which effects similar results as administration of an anti-IGFBP-2 antibody. An effective amount is described, generally, as that amount sufficient to detectably and repeatedly ameliorate, reduce, minimize or limit the extent of a disease or its symptoms. More rigorous definitions may apply, including elimination, eradication or cure of disease. Target cancer cells include cancers of the lung, brain, prostate, kidney, liver, ovary, breast, skin, stomach, esophagus, head and neck, testicles, colon, cervix, lymphatic system and blood. Of particular interest are cancers of the prostate.

To kill cells, inhibit cell growth, inhibit metastasis, decrease tumor size and otherwise reverse or reduce the malignant phenotype of tumor cells, using the methods and compositions of the present invention, one would generally contact a patient with the IGFBP-2 directed therapy. This may be combined with surgery or compositions comprising other agents effective in the treatment of cancer. Thus it is contemplated that the IGFBP-2 directed therapy may be combined with other anti-cancer therapies or agents, such as surgery, immunotherapy, chemotherapy, radiotherapy, gene therapy, or hormone therapy. These compositions would be provided in a combined amount

effective to kill or inhibit proliferation of a cancer cell. This process may involve contacting the cells with the IGFBP-2 directed therapy and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell 5 with two distinct compositions or formulations, at the same time, wherein one composition includes the IGFBP-2 directed therapy and the other includes the second agent.

Alternatively, the IGFBP-2 directed therapy may precede or follow the other 10 agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and IGFBP-2 directed therapy are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and IGFBP-2 directed therapy would still be able to exert an advantageously combined effect on the cell. In such instances, it is 15 contemplated that one would contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other, with a delay time of only about 12 h being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective 20 administrations.

i) Chemotherapy

Cancer therapies can include a variety of combination therapies with both 25 chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatin, 5-fluorouracil, vincristin, vinblastin and methotrexate or any analog or derivative variant thereof.

30

ii) Radiation Therapy

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these
5 factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength
10 and type of radiation emitted, and the uptake by the neoplastic cells.

iii) Surgery

Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery.
15 Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

Curative surgery includes resection in which all or part of cancerous tissue is
20 physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and miscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental
25 amounts of normal tissue.

iv) Gene Therapy

In yet another embodiment, the secondary treatment is gene therapy in which a therapeutic polynucleotide is administered before, after, or at the same time as the
30 IGFBP-2 directed agent. Genes encoding proteins that induce cellular proliferation, that inhibit cell proliferation, or that regulate apoptosis are especially useful in the context of the present invention. Genes encoding proteins that induce cellular

proliferation include erbA, erbB, fos, jun, myc, src, ras, abl, fms, neu, her, myb, and rel. In one embodiment of the present invention, it is contemplated that anti-sense mRNA directed to a particular inducer of cellular proliferation is used to prevent expression of the inducer of cellular proliferation. Genes that encode inhibitors of cell 5 proliferation include genes known as tumor suppressor genes. Examples are p53, p16, Rb, CCAM, APC, DCC, NF-1, NF-2, and WT-1. Many proteins involved in apoptosis are in the Bcl family. Some of these act like Bcl-2 (*e.g.*, Bcl_{XL}, Bcl_w, Bcl_s, Mcl-1, A1, Bfl-1), while others counteract Bcl-2 function and promote cell death (*e.g.*, Bax, Bak, Bik, Bim, Bid, Bad, Harakiri). The genes encoding proteins involved in apoptosis are 10 contemplated for use with compositions of the present invention. Other genes that may be employed in an anti-cancer therapy with an IGFBP-2 directed agent include MEN-I, MEN-II, zac1, p73, VHL, MMAC1 / PTEN, DBCCR-1, FCC, rsk-3, p27, p27/p16 fusions, p21/p27 fusions, anti-thrombotic genes (*e.g.*, COX-1, TFPI), PGS, Dp, E2F, *trk*, *ret*, *gsp*, *hst*, E1A, p300, genes involved in angiogenesis (*e.g.*, VEGF, 15 FGF, thrombospondin, BAI-1, GDAIF, or their receptors) and MCC.

Finally, it is contemplated that the other IGFBP family members such as IGFBP-1, -3, -4, or -5, may be used in gene therapy methods in combination with an IGFBP-2 directed therapy. For example, the level of IGFBP-3 may be modulated in 20 conjunction with the administration of an IGFBP-2 directed therapy. Also, the level of proteins implicated in the regulation of IGFBPs, such as prostate-specific antigen (PSA) may also be altered in a combination treatment. Levels of PSA may be reduced by any of the methods described herein, such as by antisense of antibody modulation, or a vaccine against PSA may be used. See U.S. Patent No. 5,925,362, which is 25 hereby incorporated by reference. Alternatively, genes involved in IGFBP modes of action, for example, IGF-II-R, IGFs, IGF-I-R may also be similarly employed.

v) *Hormonal Therapy*

Hormonal therapy may also be used in conjunction with the present invention 30 or in combination with any other cancer therapy previously described. The use of hormones may be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones

such as testosterone or estrogen (e.g., anti-androgen or anti-estrogen agents). This treatment is often used in combination with at least one other cancer therapy as a treatment option or to reduce the risk of metastases.

5

vi) Other Anti-Cancer Therapies

Another form of therapy for use in conjunction with chemotherapy, radiation therapy or biological therapy includes hyperthermia, which is a procedure in which a patient's tissue is exposed to high temperatures (up to 106°F). External or internal heating devices may be involved in the application of local, regional, or whole-body 10 hyperthermia. Local hyperthermia involves the application of heat to a small area, such as a tumor. Heat may be generated externally with high-frequency waves targeting a tumor from a device outside the body. Internal heat may involve a sterile probe , including thin, heated wires or hollow tubes filled with warm water, implanted microwave antennae, or radiofrequency electrodes.

15

A patient's organ or a limb is heated for regional therapy, which is accomplished using devices that produce high energy, such as magnets. Alternatively, some of the patient's blood may be removed and heated before being perfused into an area that will be internally heated. Whole-body heating may also be implemented in 20 cases where cancer has spread throughout the body. Warm-water blankets, hot wax, inductive coils, and thermal chambers may be used for this purpose.

Various combinations may be employed, for instance, where the IGFB-2 directed agent is "A" and the second anti-cancer therapy is "B":

25

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B

B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

30

B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell
5 in a combined amount effective to kill the cell or prevent it from dividing.

Administration of the IGFBP-2 directed therapy of the present invention to a patient will follow general protocols for the administration of chemotherapeutics, taking into account the toxicity, if any, of the therapies. It is expected that the
10 treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described IGFBP-2 directed therapies.

Depending on the particular cancer to be treated, administration of therapeutic
15 compositions according to the present invention will be *via* any common route so long as the target tissue is available *via* that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Topical administration would be particularly advantageous for treatment of skin cancers. Alternatively, administration will be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection.
20 Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients.

In certain embodiments, *ex vivo* therapies also are contemplated. *Ex vivo*
25 therapies involve the removal, from a patient, of target cells. The cells are treated outside the patient's body and then returned. One example of *ex vivo* therapy would involve a variation of autologous bone marrow transplant. Many times, ABMT fails because some cancer cells are present in the withdrawn bone marrow, and return of the bone marrow to the treated patient results in repopulation of the patient with cancer
30 cells. In one embodiment, however, the withdrawn bone marrow cells could be treated while outside the patient with an viral particle that targets and kills the cancer cell. Once the bone marrow cells are "purged," they can be reintroduced into the patient.

The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. Also of importance is the subject to be treated, in particular, the state of the subject and the protection desired. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of plaque forming units (pfu) of the viral construct. Unit doses range from 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} pfu and higher.

One of the preferred embodiments of the present invention involves the use of viral vectors to deliver therapeutic genes to cancer cells. According to the present invention, one may treat the cancer by directly injection a tumor with the viral vector. Alternatively, the tumor may be infused or perfused with the vector using any suitable delivery vehicle. Local or regional administration, with respect to the tumor, also is contemplated. Finally, systemic administration may be performed. Continuous administration also may be applied where appropriate, for example, where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease. Delivery *via* syringe or catheterization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition *via* continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

For tumors of > 4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of < 4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to

about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals.

Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may
5 be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

10

In certain embodiments, the tumor being treated may not, at least initially, be resectable. Treatments with therapeutic viral constructs may increase the resectability of the tumor due to shrinkage at the margins or by elimination of certain particularly invasive portions. Following treatments, resection may be possible. Additional viral
15 treatments subsequent to resection will serve to eliminate microscopic residual disease at the tumor site.

A typical course of treatment, for a primary tumor or a post-excision tumor bed, will involve multiple doses. Typical primary tumor treatment involves a 6 dose
20 application over a two-week period. The two-week regimen may be repeated one, two, three, four, five, six or more times. During a course of treatment, the need to complete the planned dosing may be re-evaluated.

9. PHARMACEUTICAL COMPOSITIONS

25 Aqueous compositions of the present invention comprise an effective amount of the IGFBP-2 directed therapeutic dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an
30 animal, or a human, as appropriate.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. For human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

10

The biological material should be extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle, where appropriate. The active compounds will then generally be formulated for parenteral administration, e.g., formulated for injection via the intravenous, intramuscular, sub-cutaneous, intralesional, or even intraperitoneal routes. The preparation of an aqueous composition that contains an RBP agent as an active component or ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

30

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as

hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

5

An IGFBP-2 directed agent of the present invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. In terms of using peptide therapeutics as active ingredients, the technology of U.S. Patents 4,608,251; 10 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, each incorporated herein by reference, may be used.

The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, 20 phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

25 30 Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally,

dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are
5 vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparation of more, or highly, concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active
10 agents to a small area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of
15 injectable solutions described above, but drug release capsules and the like also can be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic
20 with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to
25 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.
30

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms

include, *e.g.*, tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used, including cremes.

One may also use nasal solutions or sprays, aerosols or inhalants in the present
5 invention. Nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays. Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, the aqueous nasal solutions usually are isotonic and slightly buffered to maintain a pH of 5.5 to 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic
10 preparations, and appropriate drug stabilizers, if required, may be included in the formulation. Various commercial nasal preparations are known and include, for example, antibiotics and antihistamines and are used for asthma prophylaxis.

Additional formulations which are suitable for other modes of administration
15 include suppositories and pessaries. A rectal pessary or suppository may also be used. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum, vagina or the urethra. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be
20 formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%.

Oral formulations include such normally employed excipients as, for example,
25 pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. In certain defined embodiments, oral pharmaceutical compositions will comprise an inert diluent or assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be
30 incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers,

and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 75% of the weight of the unit, or preferably between 25-60%. The amount of active compounds 5 in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following:
a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as
10 dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier.
15 Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor.

20

10. Kits

Modulators of IGFBP-2 dependent cells can be assembled into kits. These kits can contain antibody compositions that recognize IGFBP-2, recombinant vectors containing a promoter operably linked to a nucleic acid segment that encodes IGFBP-
25 2, or any other modulators as described or anticipated in this application. All the essential materials and reagents required for inhibiting an IGFBP-2 dependent tumor cell may be assembled together in a kit. When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being particularly preferred.

30

For *in vivo* use, a chemotherapeutic agent may be formulated into a single or separate pharmaceutically acceptable syringeable composition. In this case, the

container means may itself be an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the lungs, injected into an animal, or even applied to and mixed with the other components of the kit.

5

The components of the kit may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means. The kits of the invention may also include an instruction sheet defining administration of the gene therapy and/or the chemotherapeutic drug.

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, e.g., injection or blow-molded plastic containers into which the desired vials are retained. Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalant, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

11. Methods for Screening Active Compounds

The present invention also contemplates the use of IGFBP-2 dependent cells, IGFBP-2 and active fragments, and nucleic acids coding thereof, in the screening of compounds for activity in inhibiting an IGFBP-2 dependent cell. These assays may make use of a variety of different formats and may depend on the kind of "activity" for which the screen is being conducted. Contemplated functional "read-outs" include binding to a compound, inhibition of binding to a substrate, ligand, receptor or other binding partner by a compound, phosphatase activity, anti-phosphatase activity, phosphorylation of IGFBP-2, dephosphorylation of IGFBP-2, inhibition or stimulation of cell-to-cell signaling, growth, metastasis, cell division, cell migration, soft agar

colony formation, contact inhibition, invasiveness, angiogenesis, apoptosis, tumor progression or other malignant phenotype.

A. IN VITRO ASSAYS

5 In one embodiment, the invention is to be applied for the screening of compounds that bind to the IGFBP-2 peptide or polypeptide. The polypeptide or fragment may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the polypeptide or the compound may be labeled, thereby permitting determining of binding.

10

In another embodiment, the assay may measure the inhibition of binding of IGFBP-2 to a natural or artificial substrate or binding partner. Competitive binding assays can be performed in which one of the agents (IGFBP-2, binding partner or compound) is labeled. Usually, the polypeptide will be the labeled species. One may 15 measure the amount of free label versus bound label to determine binding or inhibition of binding.

20 In another embodiment, the assay may measure the inhibition of IGFBP-2 transcription, translation or secretion. These assays will generally be cell-based assays where detection of the secreted IGFBP-2 is monitored. A decrease in IGFBP-2 in the presence of a candidate substance compared to the levels of IGFBP-2 in the absence of a candidate substance would be screened for.

25 Another technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with IGFBP-2 and washed. Bound polypeptide is detected by various methods.

30 Purified IGFBP-2 can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to the polypeptide can be used to immobilize the polypeptide to a solid phase. Also,

fusion proteins containing a reactive region (preferably a terminal region) may be used to link the IGFBP-2 active region to a solid phase.

Various cell lines containing wild-type or natural or engineered mutations in
5 IGFBP-2 can be used to study various functional attributes of IGFBP-2 and how a candidate compound affects these attributes. Methods for engineering mutations are described elsewhere in this document, as are naturally-occurring alleles of IGFBP-2. In such assays, the compound would be formulated appropriately, given its biochemical nature, and contacted with a target cell. Depending on the assay, culture
10 may be required. The cell may then be examined by virtue of a number of different physiologic assays. Alternatively, molecular analysis may be performed in which the function of IGFBP-2, or related pathways, may be explored. This may involve assays such as those for protein expression, enzyme function, substrate utilization, phosphorylation states of various molecules including IGFBP-2, cAMP levels, mRNA
15 expression (including differential display of whole cell or polyA RNA) and others.

B. IN VIVO ASSAYS

The present invention also encompasses the use of various animal models. Here, the identity seen between human and rat IGFBP-2 provides an excellent
20 opportunity to examine the function of IGFBP-2 in a whole animal system where it is normally expressed. By developing or isolating mutant cells lines that fail to express or overexpress normal IGFBP-2, one can generate cancer models in rats that will be highly predictive of cancers in humans and other mammals. These models may employ the orthotopic or systemic administration of tumor cells to mimic primary and/or
25 metastatic cancers. Alternatively, one may induce cancers in animals by providing agents known to be responsible for certain events associated with malignant transformation and/or tumor progression. Finally, transgenic animals (discussed below) that lack a wild-type IGFBP-2 may be utilized as models for cancer development and treatment.

30

Treatment of animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any

route that could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated are 5 systemic intravenous injection, regional administration via blood or lymph supply and intratumoral injection.

Determining the effectiveness of a compound *in vivo* may involve a variety of different criteria. Such criteria include, but are not limited to, survival, reduction of 10 tumor burden or mass, arrest or slowing of tumor progression, elimination of tumors, inhibition or prevention of metastasis, increased activity level, improvement in immune effector function and improved food intake.

15 **C. RATIONAL DRUG DESIGN**

The goal of rational drug design is to produce structural analogs of biologically active polypeptides or compounds with which they interact (agonists, antagonists, inhibitors, binding partners, *etc.*). By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different 20 susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for IGFBP-2 or a fragment thereof. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches. An alternative approach, "alanine scan," involves the random replacement of residues throughout molecule with alanine, 25 and the resulting effect on function determined.

It also is possible to isolate a IGFBP-2 specific antibody, selected by a functional assay, and then solve its crystal structure. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to 30 bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotype would be expected to be an analog of the original

antigen. The anti-idiotype could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

5

Thus, one may design drugs which have improved IGFBP-2 activity or which act as stimulators, inhibitors, agonists, antagonists or IGFBP-2 or molecules affected by IGFBP-2 function. By virtue of the availability of cloned IGFBP-2 sequences, sufficient amounts of IGFBP-2 can be produced to perform crystallographic studies.

10 In addition, knowledge of the polypeptide sequences permits computer employed predictions of structure-function relationships.

12. BIOLOGICAL FUNCTIONAL EQUIVALENTS

As modifications and changes may be made in the structure of IGFBP-2 genes 15 and proteins of the present invention, and still obtain molecules having like or otherwise desirable characteristics, such biologically functional equivalents are also encompassed within the present invention.

For example, certain amino acids may be substituted for other amino acids in a 20 protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies, binding sites on substrate molecules or receptors, DNA binding sites, or such like. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein 25 sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein with like (agonistic) properties. It is thus contemplated by the inventors that various changes may be made in the sequence of IGFBP-2 proteins or polypeptides, or underlying DNA, without appreciable loss of their biological utility or activity.

30 In terms of functional equivalents, it is well understood by the skilled artisan that, inherent in the definition of a "biologically functional equivalent protein or peptide or gene", is the concept that there is a limit to the number of changes that may be made

within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted.

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In particular, where shorter length peptides are concerned, it is contemplated that fewer amino acid substitutions should be made within the given peptide. Longer domains may have an intermediate number of changes. The full length protein will have the most tolerance for a larger number of changes. Of course, a plurality of 10 distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention.

It also is well understood that where certain residues are shown to be particularly important to the biological or structural properties of a protein or peptide, 15 e.g., residues in binding regions or active sites, such residues may not generally be exchanged. This is an important consideration in the present invention, where changes in the IGFBP-2 should be carefully considered and subsequently tested to ensure maintenance of biological function, where maintenance of biological function is desired. In this manner, functional equivalents are defined herein as those peptides 20 which maintain a substantial amount of their native biological activity.

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid 25 side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically 30 functional equivalents.

To effect more quantitative changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); 5 alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive 10 biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ± 2 is 15 preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biological 20 functional equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in certain embodiments of the present invention. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.* with a biological 25 property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 \pm 1); glutamate (+3.0 \pm 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine 30 (0); threonine (-0.4); proline (-0.5 \pm 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

While discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes may be effected by alteration of the encoding DNA; taking into consideration also that the genetic code is degenerate and that two or more codons may code for the same amino acid. A table of amino acids and their codons is presented herein for use in such embodiments, as well as for other uses, such as in the design of probes and primers and the like.

In addition to the IGFBP-2 peptidyl compounds described herein, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure or to interact specifically with, for example, substrates or receptor compounds. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and hence are also functional equivalents.

20

Certain mimetics that mimic elements of protein secondary structure are described in Johnson *et al.* (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orientate amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is thus designed to permit molecular interactions similar to the natural molecule.

13. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to

constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5

EXAMPLE 1

The Effect of IGF, IGFBP-2 and Antibodies to IGFBP-2 on Growth and Gene Expression in Prostate Cell Lines

10 **A. MATERIALS AND METHODS**

Reagents: Rat IGFBP-2 is purchased from Research Diagnostics, Inc. (Flanders, NJ), and rat IGFBP-2 antibody is custom-made for the experiments by Genemed Synthesis Inc. (San Francisco, CA). A polyclonal antibody against human IGFBP-2 was generated using a peptide of the following sequence:
15 **LFYNEQQEARGVHTQRMQ (SEQ ID NO:3).**

Cell counting: 0.3 X10⁶ cells are plated in 60-mm dishes (GIBCO) in 5 ml medium containing 10% fetal calf serum (FCS). After overnight culture, cells are grown in serum-free medium for 24 h, and then stimulated with various concentrations 20 of IGF, IGFBP-2 and antibody to IGFBP-2 for 48 h. Control cells are incubated with control (non-immune) rabbit IgG. At term, cells will be dispersed with trypsin-EDTA solution and counted with the help of the Coulter Electronic particle counter (Model ZF, Coulter Electronics, Hialeah, FL).

25 **MTT assay:** 6,000 cells are seeded/well in 96-well plates and grown overnight in 10% FCS. Cells are maintained under serum-free conditions for 24 h, and then stimulated for 48 h with various concentrations of IGF, IGFBP-2 and antibody to IGFBP-2. The final cell number is determined with the help of MTT dye (Sigma M-30
30 2128) (Singh *et al.*, 1994). The plates are read immediately at 540 nm on a scanning multi-well spectrophotometer (Model Vmax: Molecular Devices, Melno Park, CA). Optical absorbance reading from test wells is corrected for basal control absorbance values from cell-free wells containing culture media only.

5 **Clonogenic assay:** Cells are seeded at equal concentrations (8,000 cells/well) in 6-well culture plates in 0.3% agar in growth medium containing IGF, IGFBP-2 and antibody to IGFBP-2 (MacPherson and Montagnier, 1964). The colonies are stained
with methylene blue, and total number of colonies (> 1 mm in size)/well is counted
with the help of an inverted-light microscope.

10 **RNA Extraction:** Total RNA is extracted from cells by a single-step phenol and guanidinium thiocyanate extraction method, using RNA Stat 60 (Tel-Test, Inc., Friendswood, TX). Total RNA is quantitated by Spectrophotometric readings at wavelengths of 260/270/280.

15 **RT-PCR:** cDNA is prepared from 1 microgram of total RNA, using AMV reverse transcriptase (Promega). Ten percent of the cDNA prepared is used for amplification by PCR, (30-32 PCR cycles), using appropriate primers. The PCR reaction is performed in 50 microliters buffer containing 2 microliters cDNA, 20 pmol of each primer, 250 uM dNTPs, and 0.3 units Taq polymerase. The reactions are denatured at 94°C for 30 sec, annealed at 60°C for 45 sec, and elongated at 72°C for 1 min/cycle. Twenty microliters of mixture from each reaction is then electrophoresed on
20 1% agarose and visualized with ethidium bromide staining.

25 **Analysis of RNA by Northern Hybridization:** About 10 micrograms of RNA per sample is electrophoresed through formaldehyde-agarose gels, transferred to nylon membranes, and cross-linked either with ultraviolet light or baking. Partial length complementary probes (cDNA) are used for analysis of mRNA. The cDNA probes are labeled by nick translation, using commercial kits from Promega (Madison, WI), and then hybridized to membranes using standard conditions. Membranes are washed, briefly air-dried, exposed to X-ray film for an appropriate amount of time for the signal to develop, and the autoradiograms analyzed.

B. RESULTS

RNA samples prepared from LNCap and DU145 prostate cancer cell lines were analyzed for expression of IGFBP-2 mRNA transcripts by RT-PCR. A DNA fragment consistent with the predicted size (442 bp) was present from the cells that were 5 analyzed, demonstrating that message for IGFBP-2 is present in these cell lines.

The effect of IGFBP-2 and IGFBP-2 Ab on the growth of LNCap and DU145 prostrate cancer cells was then examined. LNCap and DU145 cells were treated with different doses of IGFBP-2 or IGFBP-2 Ab and growth was measured by counting the 10 total number of cells. Control cells are grown in 1%, 5% or 10% concentrations of Fetal Calf Serum (FCS) while experimental cells were grown under serum free conditions with the indicated additives. FIG. 1A shows the effects of either 125, 250 or 500 ng/ml IGFBP-2 or 125, 250 or 500 ng/ml IGFBP-2 antibodies on the growth of LNCap cells as compared to control cells grown in 1%, 5% or 10% FCS. FIG. 15 1B shows the effects of these same treatments on DU145 cells. While IGFBP-2 stimulated the growth of both cell lines, antibodies to IGFBP-2 inhibited the growth of both cell lines.

FIG. 1C and FIG. 1D demonstrate the effects of IGFBP-2 or antibodies to 20 IGFBP-2 in the presence or absence of IGF-I (250 ng/ml). An MTT assay was used to measure relative cell numbers. Again, antibodies to IGFBP-2 inhibited the growth of both LNCap and DU145 cells. In addition, this inhibition was still observed in the presence of exogenous IGF-1.

25 A clonogenic assay was used to test the growth of DU145 cells treated with different doses of IGFBP-2 Ab. All cultures were maintained in 10% serum and treatments were administered for 5 weeks. Colonies were stained with 1% methylene blue, and colonies were counted and their relative size determined. While IGFBP-2 treatment (250 ng/ml) resulted in larger colonies compared to control cultures, 30 culturing cells in increasing concentrations of antibodies to IGFBP-2 (125, 250 and 500 ng/ml) resulted in smaller and fewer colonies compared to control cultures.

Tumorigenic potential of DU145 cells grown in the presence of IGFBP-2 or IGFBP-2 Ab was determined in a soft agar clonogenic assay (MacPherson and Montagnier, 1964). Results of colony number are shown in FIG. 2. Cells were cultured in either 1, 5 or 10% serum alone (Serum samples, FIG. 2.) or serum free 5 with 125, 250, or 500 ng/ml IGF-1, IGFBP-2 or antibodies to IGFBP-2. The total number of colonies/well in wells seeded with IGFBP-2 Ab was less than 1-5% of the numbers in wells seeded with IGFBP-2.

RT-PCR analysis was used to determine the relative levels of IGF-I mRNA in 10 LNCap cells treated with IGFBP-2 Ab. RNA samples from LNCap cells growing in culture for 3 days in the presence of varying concentrations of IGFBP-2 Ab (125, 250 or 500 ng/ml) were subjected to RT-PCR for 32 cycles, using the primers specific for hIGF-I cDNA. Ethidium bromide staining of the reaction products was used to evaluate the relative density of IGF-1 PCR products from the three control cultures 15 compared to the experimental cultures. The relative density of IGF-I message following IGFBP-2 Ab treatment was decreased approximately 50-100%, compared with the relative density of IGF-1 message in control samples.

Prostate cancer cells are treated with either IGFBP-2 or IGFBP-2 Ab and the 20 resultant changes in the expression of IGF system components (viz. IGFs, IGF-1R, and IGFBP-2) is examined. Expression of prostate cancer specific Prostate Tumor Inducing-1 (PTI-1) gene is also examined. The inventors are measuring IGF-I RNA and PTI-1 RNA levels using RT-PCR in response to IGFBP-2 Ab treatment. They are also examining levels of IGF-II and IGF-IR by RT-PCR and Northern 25 hybridization/RPA, as well as receptor binding assays.

The inventors examined the effect of IGFBP-2 on the growth of prostate cancer cell lines *in vitro*, and investigated the probable mechanism by which IGFBP-2 exerts its growth-modulatory effects. The results of these studies suggest that IGFBP- 30 2 strongly stimulates the growth of androgen-sensitive (LNCap) and androgen-independent (DU145) prostatic cancer cell lines. Growth of the cells is significantly inhibited by IGFBP-2 Antibody (Ab). The probable mechanism by which IGFBP-2 Ab

inhibited growth was by down-regulating mRNA levels of IGF-I and Prostate Tumor Inducing-1 (PTI-1) genes. Since PTI-1 is a marker for prostate cancer (Shen *et al.*, 1995; Sun *et al.*, 1997), down-regulation of mRNA levels of PTI-1 indicated cancer suppression.

5

Circulatory levels of IGFBP-2 have been reported to correlate directly with the stage and severity of cancer in prostate cancer patients. Thus, circulatory levels of IGFBP-2 may be a biomarker for progression of prostate cancer. The role of IGFBP-2 in the initiation and progression of prostate cancer is not known to date. The present 10 application demonstrates that IGFBP-2 strongly stimulated the growth of prostate cancer cell lines grown in tissue culture dishes, while suppression of IGFBP-2 significantly reversed these growth effects. Treatment with IGFBP-2 Ab resulted in reduced expression of IGF-I, which is a growth stimulating factor for prostate cells. This demonstrates that growth of prostate cancer cells can be potentiated by IGFBP-2, 15 and significantly, retarded by suppression of endogenous IGFBP-2. The inventors speculate that IGFBP-2 promotes prostate cancer in the elderly by stimulating IGF-I. Normally, the serum levels of IGF-I progressively decline due to aging. Abnormal stimulation of IGF-I by high circulatory levels of IGFBP-2 could therefore induce malignant transformation and stimulate the expression of the prostate-cancer specific 20 PTI-1 gene in the prostate of older men.

EXAMPLE 2

Effects of Stable Expression of IGFB-2 or Antisense IGFB-2 in Prostate Cell Lines

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The inventors used stably transfected clones of aggressive (DU145) and non-aggressive (LNCap) prostate cancer cell line that are over-expressing either antisense (AS) or sense (S) IGFBP-2. Growth of cells transfected with the empty vector will serve as control (C). The growth potential and clonogenic potential of stably 30 transfected S, AS and C clones of LNCap and DU145 prostate cancer cells will be measured *in vitro*. The inventors expect that S LNCap and DU145 cells will show increased clonogenic and growth potential, compared to that of the C clones, while the

clonogenic potential of AS LNCap and DU145 cells will be significantly reduced. The inventors will measure the concentrations of the IGF factors and the PTI-1 gene products at the mRNA and protein levels in the transfected cells.

5 A. MATERIALS AND METHODS

Vectors: The inventors are using the mammalian expression vector, pCEP4 (Invitrogen), for the studies. This vector uses the Epstein-Barr virus origin of replication, and has Epstein-Barr virus-encoded nuclear antigen-1 (EBNA-1), which allows it to replicate extra-chromosomally in the nuclei of eukaryotic cells. It has a
10 hygromycin B gene, which allows for selection in eukaryotic cells, and a pBR322 origin of replication, which allows growth and maintenance in *Escherichia coli* cells. It uses the cytomegalovirus promoter to drive expression of the insert in a constitutive manner.

15 Plasmids (pBluescript) containing either a partial length cDNA (503 bps, for constructing AS plasmids), or a full-length cDNA (for constructing S plasmids) of hIGFBP-2 are constructed using hIGFBP-2 cDNA clones as described (Shimasaki and Ling, 1991). The appropriate cDNA fragment of hIGFBP-2 is cloned in the sense (S) or anti-sense (AS) direction in pCEP4 vectors. The directional cloning of the cDNA
20 insert is confirmed by restriction mapping and DNA sequencing (Wood *et al.*, 1993). Using lipofectamine, DU145 and LNCap cells are transfected with the AS and S pCEP4 vectors, while Control (C) cells are transfected with only the pCEP4 vectors (no insert). Cells are allowed to recover for 2 days in non-selective medium.
25 Hygromycin (Sigma Chemical Co.) is then added at the optimal concentration (predetermined to be 1 mg/ml for both the cell lines). Resistant clones are selected by the limited dilution method, and at least 4-6 single-cell clones of the cell line, containing either the C, AS or S vectors, are further expanded under continued hygromycin selection. Functionality of the clones is confirmed by Western blot analysis of the conditioned media (CM).

30

Analysis of growth, clonogenic and tumorigenic potential: The inventors are analyzing the growth of non-transfected and transfected (S, AS and C) cells either

by measuring the total cell number at the end of the experimental time point or using a semiautomatic tetrazolium-based clonogenic (MTT) assay as described in Example 1. The tumorigenic potential of the cells *in vitro* are analyzed by a soft agar clonogenic assay (MacPherson and Montagnier, 1964).

5

Receptor Binding Assay for studying the expression of IGF-IR: S, AS and C clones of LNCap and DU145 cells are subcultured in 160 mm flasks and grown to subconfluence in culture medium containing 10% FCS. Cells in culture are washed with Hank's balanced salt solution (HBSS, Gibco, Grand Island, NY) containing 0.1% bovine serum albumin and 25 mmol/L HEPES (Sigma). Cells are scraped with a rubber policeman, collected in conical tissue culture polystyrene tubes, centrifuged and resuspended in HBSS at a concentration of 2-3 X 10⁶ cells/ml. Binding assays are performed by incubating 0.5 ml aliquots of suspended cells with 0.01 nmol/L ¹²⁵I-IGF-I (Amersham, Arlington Heights, IL) in the presence or absence of radio-inert IGF-I, at 16°C for 6 hours, at pH 8 in a shaking water bath. At the end of the incubation, the cells are pelleted, washed with fresh ice-cold HBSS plus 0.1% BSA, and counted for ¹²⁵I in a gamma-counter (model 5500, Beckman, Palo Alto, CA). The binding affinity and the total binding capacity of IGF-I to prostate cancer cells is determined from a Scatchard plot of the specific binding data. The specificity of the binding of IGF-I to the prostate cancer cells will be determined from a log-dose inhibition of specific binding of radioactive IGF-I by non-radioactive IGF-I, in terms of ED₅₀ and relative binding affinity (Singh *et al.*, 1991).

B. RESULTS

25 Autoradiographs of immunoblots of CM samples from AS and C clones of LNCap cells. Serum-free CM was collected from LNCap cells following 7 days of culture. The CM samples were concentrated and loaded in each lane (equivalent to 10⁷ cells) and analyzed by Western immunoblot analysis (WIB) with specific anti-IGFBP-2 antibody. Samples from several different clones were analyzed. IGFBP-2 (BP2) was used as a positive control for the samples. The molecular mass of the bands (35,000 kDa) was matched with that obtained from recombinant IGFBP-2.

Comparisons of IGFBP-2 signals from the different clones was used to assess the degree of overexpression under downregulation of IGFBP-2.

Growth of prostate cancer cells was examined following suppression of
5 endogenous IGFBP-2 in LNCap and DU 145 cells by transfecting them with IGFBP-2 antisense DNA. Growth of AS- clones of LNCap cells in the presence of different concentrations of serum is shown in FIG. 3. Growth was measured in terms of optical density (O.D.) by an MTT assay. Results are mean \pm SEM of 8 observations with the representative clones. A direct and positive correlation between the level of expression
10 of endogenous IGFBP-2 and the growth response of the antisense cells to varying concentrations of serum was noted, demonstrating that growth of prostate cancer cells can be inhibited by suppression of endogenous IGFBP-2.

The relative levels of IGF factors and PTI-I RNA will also be examined in the
15 AS and S clones and compared to that in the C clones of LNCap and DU145 cells. Based on the strength of our preliminary studies, we expect that we will confirm a mediating role for one or more IGF related factors in the growth-potentiating effects of IGFBP-2. Then we expect to measure significantly higher levels of the indicated IGF factors in S clones, and significantly lower levels in AS clones, compared to that in C
20 clones.

EXAMPLE 3

Effects on Prostate Cancer in an *in vivo* Animal Model Using IGFBP-2 or an Antibody to IGFBP-2

25

In this example, the inventors examine the effect of IGFBP-2 and IGFBP-2 Ab treatment on the rate of spontaneous development of prostate cancer in male retired breeders of the Copenhagen strain of rats, in which spontaneous prostatic tumors are known to develop at 22 months age (Dunning, 1963).

30

A. MATERIALS AND METHODS:

Measuring circulatory levels of IGFBP-2 by RIA: IGFBP-2

immunoreactivity is measured in 50 microliter serum samples of Copenhagen rats, using rat IGFBP-2 kit (Diagnostics Systems Laboratories, Inc., Webster, TX). Immunoprecipitates obtained in the double antibody coated RIA tubes of the kit are 5 pelleted after 4 hrs of incubation at room temperature, and radioactivity is measured in a gamma counter.

Analysis of histopathological changes: The prostate glands are dissected out and fixed in Bouin's fixative. Following routine histological procedures the glands are 10 embedded in paraffin and 5 micron sections are stained with hematoxylin and eosin, mounted with permount and observed under the microscope for microanatomical changes, as described by Noble (1982).

Examination of IGFBP-2 expression by immunocytochemical methods:

15 IGFBP-2 is detected immunocytochemically on 5 micron paraffin sections, following a standardized procedure for immunocytochemistry (Chatterjee *et al.*, 1996; Fang *et al.*, 1998), and by using 3,3'-diaminobenzidine (DAB) as the chromogen. Sections are counterstained with hematoxylin, dehydrated in alcohol and mounted in Permount for observation under the light microscope. Control sections are processed in parallel with 20 the omission of primary antibodies. A rough estimate of percentage of cells positive for IGFBP-2 and an estimate of the intensity of IGFBP-2 staining is obtained by video image analysis of the cells using a video image analysis system (Nikon Inc., Torrence, CA).

25 **Protein Extraction:** Frozen prostate glands of rats of the different age groups, and tumors generated in nude mice (Example 4) are weighed and homogenized in an ice-cold NP-40 lysis buffer (3 ml of lysis buffer/gram of tissue). The lysates are centrifuged to remove cellular debris. Protein content in the cell lysate will be estimated by the method of Lowry *et al.* (1951), using a BSA-Protein Analysis Kit 30 from Pierce Laboratories (Rockford, IL).

Western Immunoblot Analysis: Standard procedures for Western immunoblotting will be applied (Singh *et al.*, 1996). Briefly, 40-60 micrograms of the whole cell lysate is subjected to SDS 10% polyacrylamide gel electrophoresis at 35 mA for 5 h. Following electrophoresis, proteins are electroblotted onto PVDF (millipore) 5 membranes (0.5) using a Trans-Blot apparatus (BioRad). The membranes are then incubated overnight at 4°C with appropriate rat primary antibodies of the IGF system, and later with the appropriate horseradish peroxidase-conjugated secondary antibody (Research Diagnostics, Inc.). IGFBP-2 is visualized on the blots by incubating with chemiluminescence detection reagents using the ECL kit (Amersham, Arlington Heights, IL) following the manufacturer's instructions. The intensity of the bands in 10 the autoradiograms is compared to detect changes in the expression of the IGF factors of interest.

RNA Extraction: Frozen prostate glands obtained from rats of different age 15 groups, and tumors generated in nude mice (Example 4) are homogenized, and total RNA extracted by a single-step phenol and guanidinium thiocyanate extraction method, using RNA Stat 60 (Tel-Test, Inc., Friendswood, TX). Total RNA is quantitated by Spectrophotometric readings at wavelengths of 260/270/280.

RT-PCR: cDNA is prepared from 1 microgram of total RNA, using AMV 20 reverse transcriptase (Promega). 10 % of the cDNA prepared is used for amplification by PCR, (30-32 PCR cycles), using appropriate primers. The PCR reaction is performed in 50 microliters buffer containing 2 microliters cDNA, 20 pmol of each primer, 250 uM dNTPs, and 0.3 units Taq polymerase. The reactions are denatured at 25 94°C for 30 sec, annealed at 60°C for 45 sec, and elongated at 72°C for 1 min/cycle. Twenty microliters of mixture from each reaction is then electrophoresed on 1% agarose and visualized with ethidium bromide staining.

Analysis of RNA by Northern Hybridization: About 10 micrograms of RNA 30 per sample is electrophoresed through formaldehyde-agarose gels, transferred to nylon membranes, and cross-linked either with ultraviolet light or baking. Partial length complementary probes (cDNA) are used for analysis of mRNA levels for IGFs, IGF-IR

or IGFBP-2. The cDNA probes are labeled by nick translation, using commercial kits from Promega (Madison, WI), and then hybridized to membranes using standard conditions. Membranes are washed, briefly air-dried, exposed to X-ray film for an appropriate amount of time for the signal to develop, and the autoradiograms analyzed.

5

B. RESULTS

The inventors are examining whether spontaneous prostatic tumors develop in male retired breeders (4,12 and 20 months old) of Copenhagen rats is effected when the rats are administered IGFBP-2 or IGFBP-2 antibody. Baseline levels of serum 10 IGFBP-2 are measured in animals of the experimental age groups. A total of eighty four rats are grouped as follows: Group I (Control) -12 rats; Group II (IGFBP-2 treated) - 36 rats; and Group III (IGFBP-2 Ab treated) - 36 rats. Osmotic pumps are used to deliver IGFBP-2 and/or IGFBP-2 Ab. Loaded Alzet minipumps with flow regulators (flow rate 2.5 l/ hr) deliver either normal physiological saline (0.9%) (Group 15 I); IGFBP-2 (Group II) or IGFBP-2 antibody (Group III), are implanted into subcutaneous pockets on the dorsal surface of the experimental animals. The daily doses of IGFBP-2 received by animals in Group II is pre-calculated at 50% (for 12 animals), 100% (for 12 animals) and 150% (for 12 animals) above the mean baseline value, and the pumps filled accordingly. Similarly, animals in Group III are 20 administered IGFBP-2 antibody at appropriately adjusted doses. Treatment will be administered for 40, 80 or 120 days. Four animals per each dose tested in Groups II and III, and 4 animals of Group I are sacrificed at each experimental time point. Animals are euthanized following exposure to carbon dioxide and quickly decapitated. Blood is collected in heparinized tubes to quantitate serum IGFBP-2 levels. The 25 prostate glands are removed, grossly checked for any abnormalities, weighed, and either processed for routine histology and immunocytochemistry, or frozen in liquid nitrogen to examine changes in the protein and mRNA levels of IGF system components (viz. IGF-I, IGF-II, IGF-IR and IGFBP-2), and PTI-1.

30 Since the Alzet minipumps to be used can effectively function for 42 days, they will be replaced every 40th day. The inventors are generating polyclonal antibodies against rat IGFBP-2, using the help of a commercial outfit (Genemed Synthesis, Inc.,

San Francisco, CA). The crude serum will be tested for the presence of rat IGFBP-2 Ab, using IGFBP-2 ELISA assays, and the titre and specificity of the Ab will be determined. The efficacy of the Ab will be tested *in vitro* on the growth potency of LNCap cells to ensure that they produce effects similar to that measured with the 5 commercially available antibody (Genemed Synthesis, Inc., San Francisco, CA).

IGFBP-2 treatment of Copenhagen rats should result in inducing pre-neoplastic/neoplastic changes in the prostate of rats at a younger age, compared to that in untreated control rats. Conversely, IGFBP-2 Ab treatment should delay the onset of 10 the spontaneous changes that are usually measured in aged Copenhagen rats (Dunning, 1963). Circulatory levels of IGFBP-2 are significantly elevated in prostate cancer patients (Ho and Baxter, 1997; Kanety *et al.*, 1993; Figueroa *et al.*, 1998). Since prostate cancer is associated with old age, elevated levels of IGFBP-2 may be involved 15 in the development of malignancy in the prostate of older men. If IGFBP-2 promotes growth of cancer cells, then IGFBP-2 Ab may prevent malignant transformations in the prostate, and act like tumor suppressors by retarding the rate of tumor development. This application demonstrates that IGF-I and IGFBP-2 may be mediating growth 20 effects in tumors, it is quite conceivable that IGFBP-2 Ab may negate tumorigenesis in much the same way as inhibitors/antibodies of IGFs and IGF-1R have been shown to for other cancers (Wang *et al.*, 1997).

EXAMPLE 4

Tumorigenic Potential in Nude Mice of Prostate Cell Lines Expressing IGFB-2 or Antisense IGFBP-2

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The inventors are testing the tumorigenic potential of aggressive (DU145) or non-aggressive (LNCap) prostate cancer cells following modulation of levels of 30 IGFBP-2 within the cells. Stably transfected DU145 and LNCap clones that over-express either the S or the AS human IGFBP-2 mRNA (Example 2) are inoculated subcutaneously on contra-lateral sides of nude (athymic) male mice (10 animals per group). Cells will be inoculated at equal concentrations, approximately $1-10 \times 10^6$ cells for DU145, and $5-10 \times 10^6$ cells for LNCap cells in 0.2 ml Hank's balanced salt

solution (HBSS), on each side. At this concentration, DU145 cells form approximately 0.5 g tumors in about 60 days in 3 month old male athymic nude mice. In the case of LNCap cells, inoculation with even 10×10^7 cells generally does not result in developing palpable tumors till 90 days of *in vivo* growth in nude mice. At term, 5 animals are euthanized by carbon dioxide exposure, and tumors removed and weighed. A small part of the tumor will be processed for routine histology, while the rest is frozen in liquid nitrogen to measure the mRNA and protein levels of the IGF factors and the PTI-1.

10 The inventors expect to measure palpable tumors in mice inoculated with the S LNCap clones, but not C LNCap clones, within 60-90 days of *in vivo* growth. An increase in the tumorigenic potential of IGFBP-2 over-expressing DU145 is also expected. Similarly, the AS DU145 clones, in which IGFBP-2 expression is suppressed, will show delay in tumor formation in nude mice, in comparison to C 15 DU145 clones. There are methods for helping ensure that LNCap cells form tumors when injected sub-dermally. LNCap cells, co-inoculated with tissue-specific mesenchymal or stromal cells (Gleave *et al.*, 1991) or Matrigel (Gleave *et al.*, 1992) develop tumors subdermally. Also, LNCap cells inoculated directly within the prostates can grow into tumors in the orthotopic environment (Gleave *et al.*, 1992). The 20 inventors will test tumorigenic growth of S LNCap cells to that of C LNCap cells in an orthotopic environment.

EXAMPLE 5

In vivo Viral Delivery of Sense or Antisense IGFBP-2

25 In this example, the inventors will generate recombinant adenoviruses and retroviruses expressing sense (S) and anti-sense (AS) IGFBP-2 and deliver these to the prostate via surgical procedures, or intra-uretherally. Retroviral or adenoviral vectors expressing S or AS IGFBP-2 will be constructed (Singh *et al.*, 1999). The inventors 30 can then deliver the S and AS expressing IGFBP-2 virus to the prostatic cells *in situ*, by using an optimal titer of the infectious viral particles (approximately 10^6 pfu/ml for retroviruses, and approximately 10^8 or 10^9 pfu/ml of adenoviruses). Delivery of the

viral particles will include: 1) intra-peritoneal (i.p.) injections (in the lower abdomen), which is likely to be least toxic, but may lack efficiency; 2) orthotopic injection into the prostatic mass bilaterally, using surgical procedures; 3) intra-urethral injections at the site of the prostate with intra-urethral cannulations. This third delivery route is the
5 method of choice to deliver adenoviruses directly to the prostate. Since adenoviruses are episomally expressing vectors, periodic injections, approximately 3 months apart will be required, for long-term studies. This may cause inflammatory responses at the surgical site. Retroviruses, on the other hand, get integrated into the host DNA, and
10 will not require repeated injections, but can only infect dividing cells, resulting in extremely poor effectiveness compared to adenoviruses.

EXAMPLE 6

Promoter Activity Of IGF-I, IGF-II and IGF-1R in Prostate Cell Lines

15 Previous results indicate correlative changes between RNA and protein levels of IGF factors in response to IGFBP-2 or IGFBP-2 Ab, suggesting changes in transcriptional activity of these genes. In order to test this possibility, the inventors are examining the promoter activity of the indicated genes (IGF-I, IGF-II, IGF-1R) by using promoter-reporter assays.

20

A. MATERIALS AND METHODS:

Transient transfection of prostate cancer cells: Full length IGF-I, IGF-1R and IGF-II promoters are cloned into promoterless luciferase reporter vectors to construct the promoter-luciferase vectors. While the promoters driving the expression
25 of hIGF-I and hIGF-1R are well defined, at least 4 promoters can be potentially used to drive the expression of IGF-II (Singh *et al.*, 1998). IGF-II promoters P3 and P4 are highly active in many cancers, while P1 and P2 are relatively silent. The inventors are confirming the activity of the four P1-P4 IGF-II promoters in the DU 145 and LNCap cells to use the appropriate promoter(s) for the present studies. The promoter-luciferase vectors are transfected to LNCap and DU145 cells. All cells are co-transfected with β -gal expression vectors to correct for transfection efficiency. After transfection, cells are maintained in serum-free media, and treated with IGFBP-2 or

IGFBP-2 Ab. Cells are lysed 24-48 h post transfection, and luciferase and β -gal activities measured, as described below.

Luciferase and β -Gal assays: Cells are washed with PBS and lysed using the reporter lysis buffer (Promega) as per the protocols provided by the company. Cellular debris is pelleted and the supernatant saved for analysis of luciferase and β -Gal activities. Luciferase activity is measured with the Luciferase Assay system (Promega), using 20 microliters of cell extract and 100 microliters luciferase assay buffer, at room temperature. Luciferase activity is measured within 15 sec of adding the substrate, luciferin, with a Turner TD-20e Luminometer (Turner Designs, Sunnyvale, CA). The β -Gal assay is performed with the β -Galactosidase Enzyme Assay System (Promega) as per the protocols provided by the company. Ninety-six well plates are used for this assay. 30 microliters of cell extracts will be mixed with 20 microliters of 1x reporter buffer and 50 microliters of 2x assay buffer in each well and incubated at 37°C for 30 min. The reactions will be stopped by adding 150 microliters of 1M sodium carbonate. Absorbance of the samples is read at 405 nm in a Umx Kinetic Microplate reader (Molecular Devices, Menlo Park, CA). The ratio of luciferase/ β -gal readings reflects the promoter activity of the indicated genes in the AS, S and C clones of prostate cancer cells.

20

B. RESULTS

The inventors expect that the changes measured in the levels of RNA and protein in response to IGFBP-2 or IGFBP-2 Ab treatment are reflected in similar changes in the transcriptional activity of the exogenous promoter-reporter plasmid in the transient transfection experiments. Taken together, these results demonstrate that IGFBP-2 can modulate the transcription of the powerful mitogenic IGF factors. The inventors studies suggest that the promoter activity of one or more IGF factors is significantly increased on treatment with IGFBP-2, and a positive correlation is established between the levels of the various IGF factors and the *in vitro* growth/clonogenic potential of the AS, S and C clones of the two cell lines.

EXAMPLE 7

Quantitation of IGF-I in *in vitro* Studies

A. MATERIAL AND METHODS

The protocols and reagents described in the previous examples are applicable
5 to this example.

B. RESULTS

The results of experiments discussed in the previous Examples suggested that
mRNA levels for IGF-I were suppressed in a dose-dependent manner following
10 treatment with IGFBP-2 antibody. The level of IGF-I protein in cell lines of treated
and untreated prostate cancer cells was evaluated. The conditioned media (CM) from
the cultures of approximately 10^7 cells of the Control and treated groups was
collected, concentrated using Amicon microconcentrators (Centricon 20, W.R. Grace,
Beverly, MA), and measured for the IGF-I and IGF-II levels by using specific RIA kits
15 (Diagnostic Systems Laboratories, Webster, TX), and following the kit instructions.
The results of the assay showed low (50 ng/ml) levels of IGF-I in the Control cultures.
Treatment with IGFBP-2 antibody resulted in undetectable levels of IGF-I in the CM
(FIG. 4). These findings strongly support my preliminary observations on the
diminution of IGF-I following administration of IGFBP-2 antibody.

20

EXAMPLE 8

Effect of Modulating IGFBP-2 *in vivo* on Tumor Initiation and Progression of Tumor Growth

25 A. MATERIAL AND METHODS

Twenty-one male athymic nude mice were divided into Control (8 animals) and
Treated (13 animals) groups. Each of the Control animals were inoculated
subcutaneously with a single injection of 2×10^6 cells mixed with 12 µg non-immune
30 IgG in 0.1 ml HBSS on the dorsal surface. The IgG was added to the cells
immediately before injection. Animals of the treated group were injected with an

equivalent number of cells mixed with anti-IGFBP-2 IgG at the same concentration, (i.e. 12 µg of anti-IGFBP-2 IgG per 2×10^6 cells).

B. RESULTS

5 Tumor Initiation

After 14 days of cell inoculation, palpable tumors were seen in 7 of 8 animals of the Control group. In striking contrast to this, tumor development was noted in only 1 out of 13 animals of the treated group (FIG. 5). Hence, anti-IGFBP-2 significantly retarded the initiation of DU145 tumorigenesis in male thymic nude mice.

10

Progression of tumor growth:

Tumor development was closely monitored in the animals used in the tumor initiation studies described above. Well-formed tumors were seen in most of the experimental animals after two months of inoculation with DU 145 cells. The 15 experiment was terminated at this time to compare the weight of the tumors in the two groups of animals. Thus, at autopsy, the tumors were carefully removed, freed from adjacent tissues, and weighed.

The mean weight of the tumors in the two groups are presented in FIG. 6. The 20 mean tumor weight of tumors generated from Control DU145 cells was $1.04 + 0.16$ grams, while that of cells treated with anti-IGFBP-2 IgG at the time of inoculation was $0.28 + 0.08$ grams. Statistical analysis of the tumor weight following student's t-test revealed that the weight of the tumors generated from wild type DU145 cells was significantly greater than those generated from anti-IGFBP-2 treated DU145 cells.

25

EXAMPLE 9**Further Studies of Progression of Tumor Growth *in vivo*****Experiment 1**5 **A. MATERIAL AND METHODS**

Twenty-one male athymic nude mice were divided into three groups. Sets of 20×10^6 DU145 aggressive prostate cancer cells were mixed with 30, 60 or 120 μg (*i.e.*, 10, 20 or 40 μl from 3 mg/ml stock) of anti-IGFBP-2 IgG. Respective groups of animals were subcutaneously inoculated on the left side of the upper arm with 2×10^6 cells in 0.1 ml PBS. The contra-lateral side was inoculated with an equal concentration of untreated DU145 cells, and served as the control. Animals were examined for tumor development after ten days of inoculation. When tumors attained a size of 1 cm across, 300 ng of IgG was administered twice a week into the tumors on the left side of tumor-bearing animals. All treatments were continued for one month. At term, the 10 tumors were carefully dissected out, weighed, and examined for gross anatomical features.

15

Group I: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 30 μg anti-IGFBP-2 IgG on 20 left.

Group II: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 60 μg anti-IGFBP-2 IgG on left.

25

Group III: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 120 μg anti-IGFBP-2 IgG on left.

Group IV: Inoculated with untreated cells (2×10^6 cells) on right; and 0.1 ml of 1 ml cell suspension of 20×10^6 cells treated with 60 µg of non-immune IgG on left.

5 Group V: Inoculated with untreated cells (2×10^6 cells) on both right and left sides.

B. RESULTS

Tumors were first evident after 16 days on the right side of animals of Groups 10 I-III. There was an apparent delay of about 6 days in the appearance of the tumor on the left side of the animals, where cells were mixed with various concentrations of anti-IGFBP-2 IgG. The size of the tumors on the left side (cells mixed with anti-IGFBP-2 IgG) was significantly smaller than tumors on the right side (untreated cells) in the experimental groups of animals (FIG. 7).

15

Experiment 2

A. MATERIAL AND METHODS

In another set of experiments, ten animals were divided into two groups (Groups A and B). Animals of Group A and B were inoculated on both sides with 2×20 10^6 DU145 cells. Tumors were evident by day 16 in both groups of animals. At this time, tumors in animals of Group A were treated with 300 ng of anti-IGFBP-2 IgG twice a week for one month, while tumors of Group B were administered 0.1 ml of saline. The experiment was terminated after one month. At autopsy, the tumors were carefully removed and weighed.

25

B. RESULTS

On comparison of the weights of the tumors, it was found that tumors in Group A animals were significantly reduced, in comparison to that in sham control animals of Group B (FIG. 8). On gross anatomical examination of the tumors, necrosis was 30 evident in most of the tumors treated with anti-IGFBP-2 IgG. Our studies suggest that IGFBP-2 antibody can significantly retard the growth of prostatic tumors in nude mice.

5 All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence
10 of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be
15 within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated
5 herein by reference.

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- U.S. Patent 4,196,265
- U.S. Patent 4,554,101
- 10 U.S. Patent 4,578,770
- U.S. Patent 4,596,792
- U.S. Patent 4,599,230
- U.S. Patent 4,599,231
- U.S. Patent 4,601,903
- 15 U.S. Patent 4,608,251
- U.S. Patent 4,683,195
- U.S. Patent 4,683,202
- U.S. Patent 4,800,159
- U.S. Patent 5,279,721
- 20 U.S. Patent 5,354,855
- U.S. Patent 5,359,046
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CLAIMS:

1. A method of inhibiting an IGFBP-2 dependent cancer cell comprising reducing IGFBP-2 activity level with an IGFBP-2 modulator.
5
2. The method of claim 1, wherein inhibiting an IGFBP-2 dependent cancer cell comprises altering proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion.
10
3. The method of claim 1, wherein said modulator decreases the amount of IGFBP-2.
15
4. The method of claim 1, wherein said modulator inhibits expression of IGFBP-
2.
5. The method of claim 1, wherein said modulator inhibits transcription of
IGFBP-2.
20
6. The method of claim 1, wherein said modulator inhibits translation of IGFBP-2.
7. The method of claim 1, wherein said modulator inhibits secretion of IGFBP-2.
25
8. The method of claim 1, wherein said modulator is an agonist or antagonist of
IGFBP-2.
9. The method of claim 1, wherein said modulator of IGFBP-2 is a nucleic acid
containing a promoter operably linked to a IGFBP-2 gene segment.
30
10. The method of claim 9, wherein the IGFBP-2 gene segment is positioned, in
reverse orientation, under the control of a promoter that directs expression of an
antisense product.

11. The method of claim 9, wherein the nucleic acid encodes a ribozyme specific for an RNA transcript of IGFBP-2 in a cell expressing an RNA transcript of IGFBP-2.
- 5 12. The method of claim 9, wherein the nucleic acid segment is located on a vector.
13. The method of claim 12, wherein the vector is a viral vector selected from the group consisting of adenovirus, retrovirus, herpes virus, adeno-associated virus, and vaccinia virus.
- 10 14. The method of claim 1, wherein said modulator is an antibody composition comprising an antibody that recognizes IGFBP-2.
15. The method of claim 14, wherein said antibody composition comprises a monoclonal antibody.
16. The method of claim 14, wherein said antibody composition comprises polyclonal antibodies.
- 20 17. The method of claim 14, wherein said antibody is a humanized antibody.
18. The method of claim 1, wherein said modulator is at least one polypeptide that interacts with IGFBP-2.
- 25 19. The method of claim 21, wherein said polypeptide comprises single chain antibodies, IGF-I, IGF-II, IFGBP-2 receptor, or a fusion protein or fragment thereof.
20. The method of claim 1, wherein said modulator is a polypeptide involved in post-translational modification of IGFBP-2.
- 30 21. The method of claim 1, wherein said cell is in a mammal.

22. The method of claim 21, wherein said mammal is a human.

23. The method of claim 1, wherein said cell is derived from the prostate.

5 24. A method of treating a subject with cancer comprising administering to said subject an effective amount of a first IGFBP-2 modulator.

25. The method of claim 24, wherein said modulator is an antibody composition comprising an antibody that recognizes IGFBP-2.

10 26. The method of claim 25, wherein said antibody composition comprises a monoclonal antibody.

15 27. The method of claim 25, wherein said antibody composition comprises polyclonal antibodies.

28. The method of claim 25, wherein said antibody is a humanized antibody.

29. The method of claim 24, wherein said modulator is a ribozyme or anti-sense sequence complementary to the IGFBP-2 gene.

20 30. The method of claim 24, wherein the subject is human.

31. The method of claim 24, wherein said treating a subject with cancer comprises killing a cancer cell, inhibiting the growth of a cancer cell, inducing apoptosis of a cancer cell, inhibiting metastatic potential of a cancer cell, reducing tumor burden, or inducing tumor regression.

25 32. The method of claim 24, wherein the cancer is prostate cancer.

30 33. The method of claim 24, further comprising treating the subject with a second agent, wherein the second agent is a second modulator of IGFBP-2, a therapeutic

polypeptide, nucleic acid encoding a therapeutic polypeptide, a chemotherapeutic agent, or a radiotherapeutic agent.

34. A method of screening a candidate substance for anti-tumor activity comprising
5 the steps of:

- (i) contacting a first IGFBP-2 dependent cell with said candidate substance; and
- (ii) comparing one or more characteristics of said first cell in the presence
10 of said candidate substance with one or more characteristics of a second IGFBP-2 dependent cell in the absence of said candidate substance.

35. The method of claim 34, wherein said characteristic is selected from the group consisting of IGFBP-2 expression, proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, and tumor invasion.
15

36. The method of claim 34, wherein said cell is contacted *in vitro*.
20 37. The method of claim 34, wherein said cell is contacted *in vivo*.

38. A pharmaceutical composition comprising a recombinant vector containing a promoter operably linked to a IGFBP-2 gene segment.
25 39. The pharmaceutical composition of claim 38, wherein the IGFBP-2 gene segment is positioned, in reverse orientation, under the control of a promoter that directs expression of an antisense product.

40. The pharmaceutical composition of claim 38, wherein the recombinant vector
30 expresses a ribozyme specific for an RNA transcript of IGFBP-2 in a cell expressing an RNA transcript of IGFBP-2.

41. A method of treating a patient with cancer comprising administering to the patient a composition comprising a peptide comprising at least 8 contiguous amino acids from SEQ ID NO:2 in an amount effective to convey a therapeutic benefit on the patient.

5

42. The method of claim 41, wherein the composition further comprises an adjuvant.

43. The method of claim 42, wherein the adjuvant and the peptide are covalently linked.

10 44. The method of claim 41, wherein the peptide comprises at least 8 contiguous amino acids from SEQ ID NO:3.

15 45. The method of claim 44, wherein the peptide comprises the amino acids of SEQ ID NO:3.

46. The method of claim 41, wherein the therapeutic benefit conveyed on the patient is inhibition of an IGFBP-2-dependent cancer cell.

20

47. The method of claim 46, wherein inhibition of an IGFBP-2 dependent cancer cell comprises altering proliferation, metastasis, contact inhibition, soft agar growth, cell cycle regulation, tumor formation, tumor progression, differentiation, or tumor invasion.

25

48. The method of claim 41, wherein the patient has a tumor.

49. The method of claim 41, wherein the cancer is prostate cancer.

30 50. The method of claim 49, wherein the therapeutic benefit conveyed on the patient is a reduction in tumor size.

51. The method of claim 41, wherein the patient is further administered chemotherapy, radiotherapy, surgery, hormone therapy, or gene therapy.

52. A method of preventing cancer in a subject comprising administering to the subject a composition comprising a peptide comprising at least 8 contiguous amino acids from SEQ ID NO:2 in an amount effective to elicit an immune response against IGFBP-2.

53. The method of claim 51, wherein the composition further comprises an adjuvant.

54. The method of claim 53, wherein the adjuvant is covalently linked to the peptide.

1/6

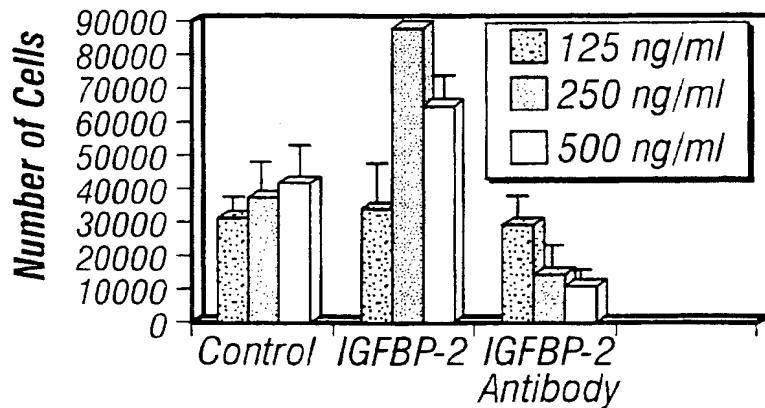


FIG. 1A

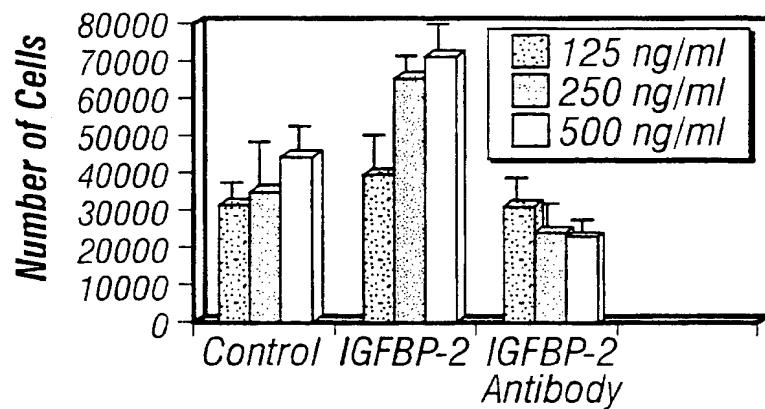


FIG. 1B

2/6

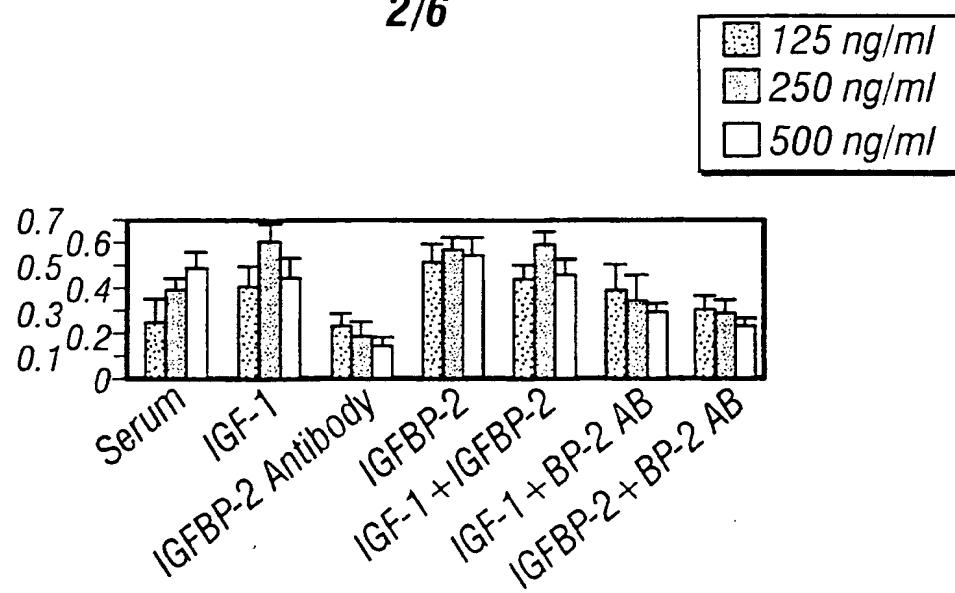


FIG. 1C

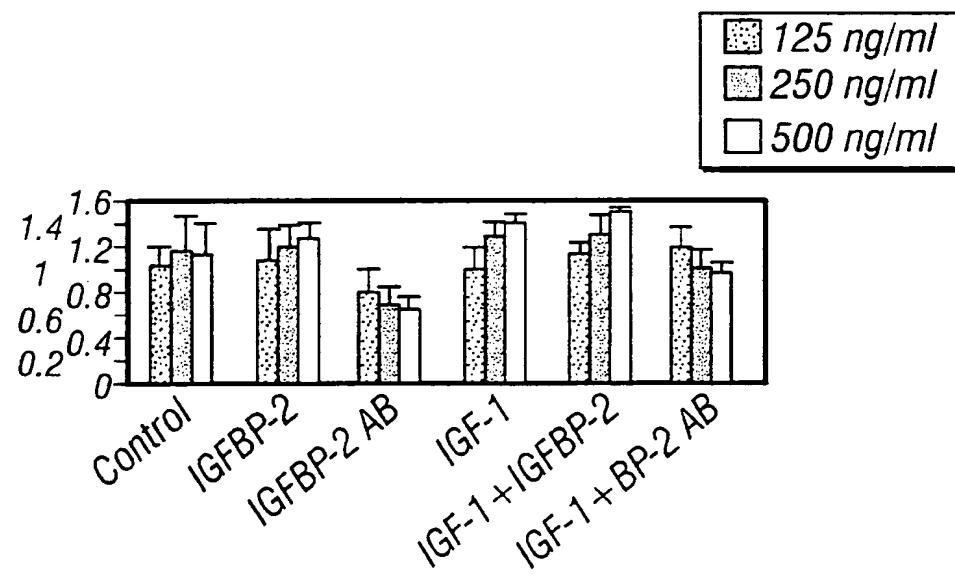


FIG. 1D

3/6

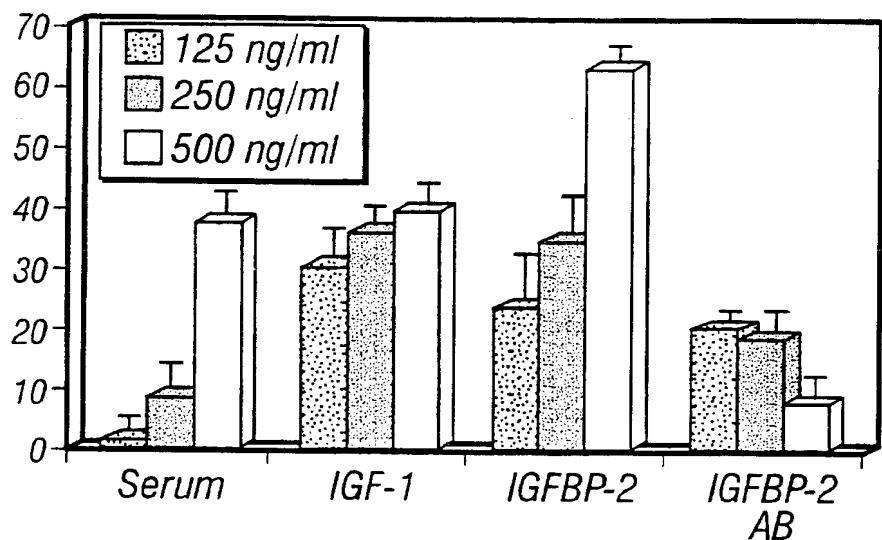


FIG. 2

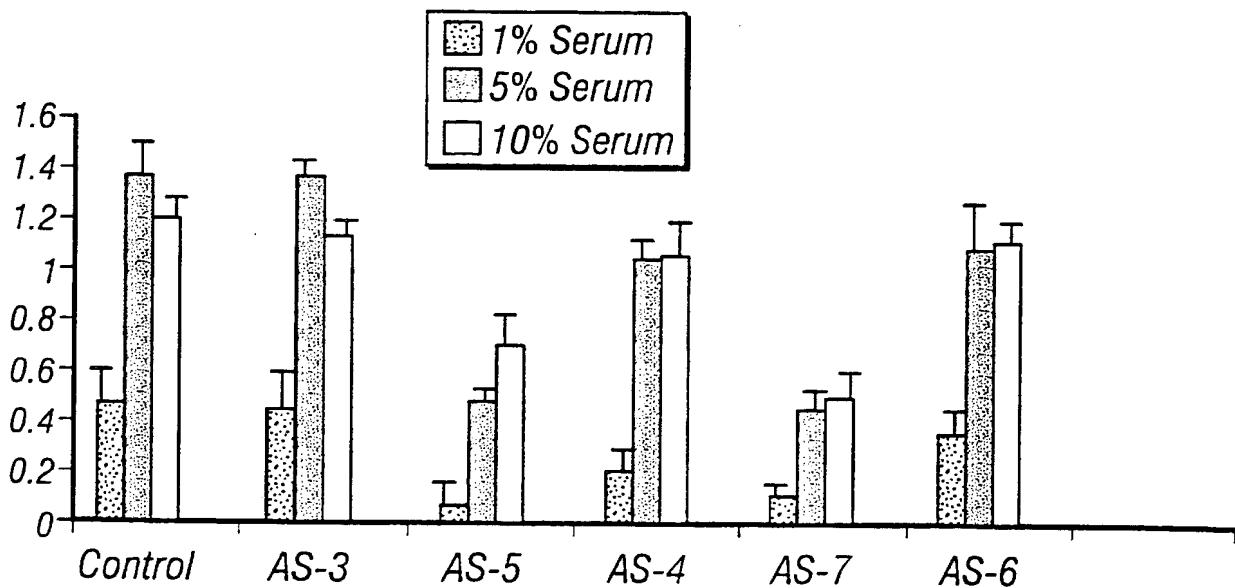


FIG. 3

4/6

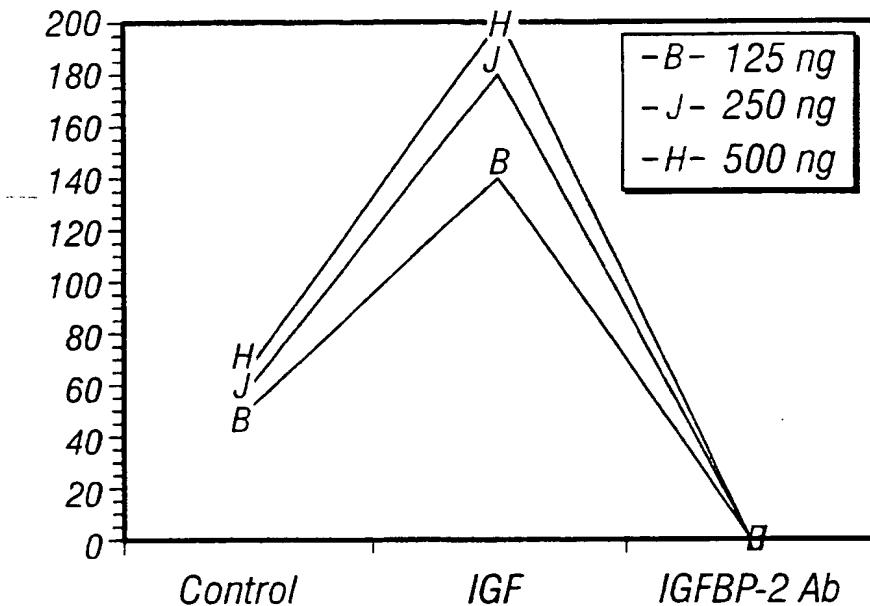


FIG. 4

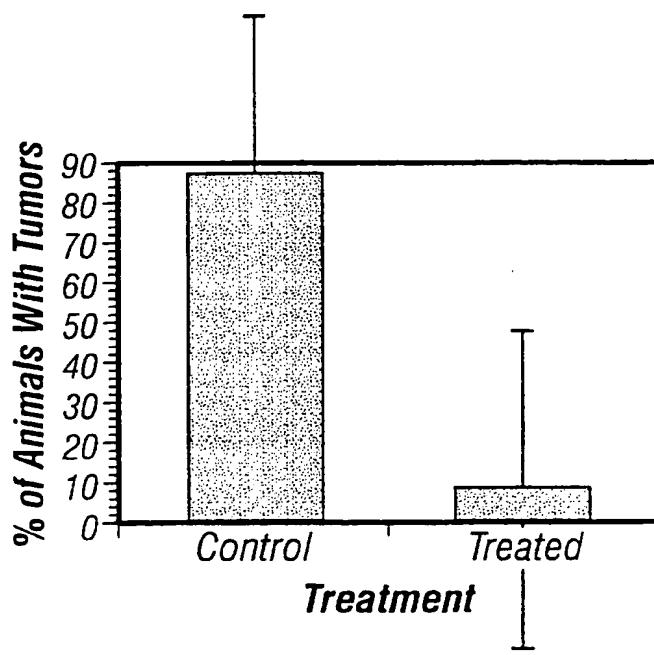


FIG. 5

5/6

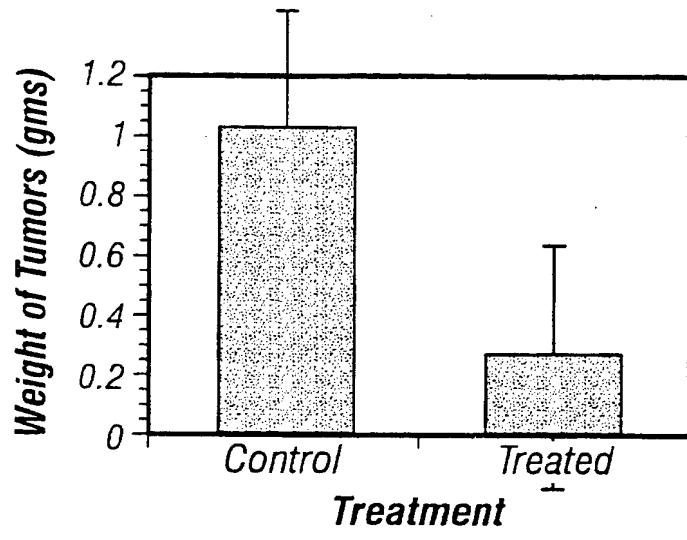


FIG. 6

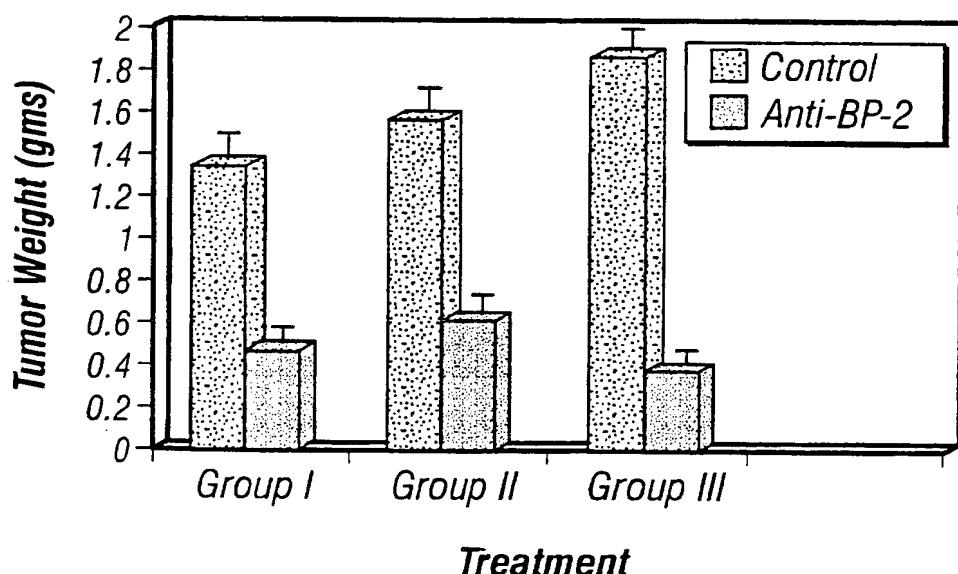


FIG. 7

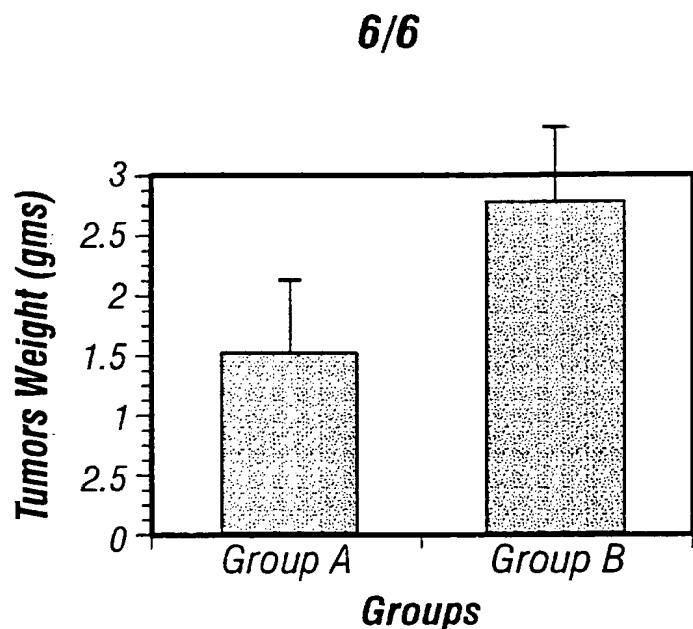


FIG. 8

SEQUENCE LISTING

<110> CHAKRABARTY, SHILLA
SINGH, POMILA

<120> SUPPRESSION OF ENDOGENOUS IGFBP-2 TO INHIBIT CANCER

<130> UTSG233

<140> FILED HEREWITH
<141> 2000-05-17

<150> 60/134,581
<151> 1999-05-17

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35 40 45
Thr Pro Glu Arg Leu Ala Ala Cys Gly Pro Pro Pro Val Ala Pro Pro
50 55 60
Ala Ala Val Ala Ala Val Ala Gly Gly Ala Arg Met Pro Cys Ala Glu
65 70 75 80
Leu Val Arg Glu Pro Gly Cys Gly Cys Ser Val Cys Ala Arg Leu
85 90 95
Glu Gly Glu Ala Cys Gly Val Tyr Thr Pro Arg Cys Gly Gln Gly Leu
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Arg Cys Tyr Pro His Pro Gly Ser Glu Leu Pro Leu Gln Ala Leu Val
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Met Gly Glu Gly Thr Cys Glu Lys Arg Arg Asp Ala Glu Tyr Gly Ala
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Ser Pro Glu Gln Val Ala Asp Asn Gly Asp Asp His Ser Glu Gly Gly
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Gly Lys His His Leu Gly Leu Glu Glu Pro Lys Lys Leu Arg Pro Pro
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245 250 255
Tyr Ser Leu His Ile Pro Asn Cys Asp Lys His Gly Leu Tyr Asn Leu
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Lys Gln Cys Lys Met Ser Leu Asn Gly Gln Arg Gly Glu Cys Trp Cys
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/13574

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/17, 18; 39/00; 48/00
US CL : 514/2, 44; 424/130.1, 158.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 44; 424/130.1, 158.1, 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, BIOSIS
search terms: IGFBP-2, cancer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FORSYTH, E. Growth inhibition of a human colon cancer cell line by antisense oligonucleotides to IGFBP-2. Gastroenterology. 1995. Vol.108, No.4, suppl. page A726.	1-10, 21-22, 34-36
A	MICHELL, N.P. Insulin-like growth factor binding proteins as mediators of IGF-I effects on colon cancer cell proliferation. Growth factors. 1997. Vol. 14, No. 4, pages 269-277.	1-54
A	MICHELL, NP. Insulin-like growth factors and their binding proteins in human colonocytes: preferential degradation of insulin-like growth factor binding protein 2 in colonic cancers. British Journal of Cancer. 1997. Vol.76, No.1, pages 60-66.	1-54

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O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

22 SEPTEMBER 2000

Date of mailing of the international search report

24 OCT 2000

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/13574

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COHEN. P. Elevated levels of insulin-like growth factor-binding protein-2 in the serum of prostate cancer patients. Journal of Clinical Endocrinology and Metabolism. April 1993. Vol.76, No.4, pages 1031-1035.	1-54
A	BOULLE. N. Increased levels of insulin-like growth factor II (IGF-II) and IGF-binding protein-2 are associated with malignancy in sporadic adrenocortical tumors. Journal of Clinical Endocrinology and Metabolism. 1998. Vol.83, No.5, pages 1713-1720.	1-54

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